ENDOGENOUS AND EXOGENOUS CONTROL OF OVARIAN DYNAMICS IN WAPITI

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ABSTRACT

A series of studies were guided by the principal hypothesis that it was necessary to characterize ovarian function during the seasons of the annual reproductive cycle in wapiti and that from this knowledge novel methods of exogenous control of ovarian function would be possible. To augment existing knowledge about endogenous control of ovarian function in wapiti studies were conducted to characterize ovarian follicle dynamics during the estrous season and to characterize ovarian follicular dynamics during the periods of transition into and out of the breeding season. The third study was designed to characterize ovarian follicle development and ovulation synchrony subsequent to an estrous synchronization protocol used commercially. To evaluate novel methods of exogenous control of ovarian function studies were conducted to determine if follicular wave emergence could be electively induced using hormonal or surgical treatments to evaluate novel ovarian superstimulatory treatment protocols.

It was concluded from the studies of ovarian function that follicle development during the breeding season was characterized by the regular and synchronous

development of follicular waves and that 2, 3, or 4 waves occurred during each interovulatory interval. Transition into the breeding season was preceded by one short interovulatory interval (9 days) characterized by one wave of follicle development and a small, short-lived and hypo-functional corpus luteum. The last estrous cycle of the breeding season was similar to estrous cycles during the rut (21 days), but and transition to anestrus was marked by a failure of the dominant follicle to ovulate after luteal regression. The treatment protocol used commercially for estrous synchronization was effective, but unnecessarily long. It was concluded from the studies on exogenous control of ovarian function that follicular wave emergence could be electively induced using steroid hormones or follicle ablation and may be useful for estrus synchronization and superstimulatory protocols. The tested superstimulatory treatments were effective and had the advantage of reducing the treatment period by 6 days and the number of times the animals are handled by one third over a more conventional method. However, oocyte and embryo quality were not evaluated.

As a result of the studies conducted and one previous study during the anouvlatory season follicle and luteal dynamics are now known in wapiti for all seasons of the year and this knowledge will provide a template upon which other species of deer can be compared. The final two studies support the principal hypothesis. The novel methods of exogenous ovarian control tested increase the potential for success when applying reproductive technologies and the successful application of these methods in wapiti should lead to their successful use in other species of deer.

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
ART	Assisted Reproductive Techniques
cAMP	cyclic Adenosine Monophosphate
cGMP	cyclic Guanosine Monophosphate
CIDR	Controlled Intervaginal Drug Releasing
CIDR-B	Controlled Intervaginal Drug Releasing – Bovine
CL	Corpus Luteum
eCG	equine Chorionic Gonadotropin
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HIOMT	Hydroxy Indole-O-MethylTransferase
im	intermuscular
IOI	InterOvulatory Interval
IU	International Units
LH	Luteinizing Hormone
MHz	MegaHertz
MOET	Multiple Ovulation and Embryo Transfer
mya	millions of years ago
NAT	N-AcetylTransferase
NZ	New Zealand

sc	subcutaneous
SCN	SuperChiasmatic Nucleus
SEM	Standard Error of the Mean
TH	Tyrosine Hydralase

1.0 GENERAL INTRODUCTION

North American wapiti are considered conspecific with the Western European red deer and the two are subspecies of *Cervus elaphus*, which represent geographical extremes of a large group with many other subspecies (Dratch & Gyllensten, 1983). *Cervus elaphus* is one of 47 species of deer present in the world today (Duff, 2004). Deer can be found in many diverse geographical regions with great variations in body size and adapted to equally varying environmental conditions. In addition deer have developed several different strategies for reproduction that are reflected in differences in their reproductive physiology. Some species of deer are strictly seasonal whereas others are aseasonal and some are usually monotocous whereas others are usually polytocous. These reproductive differences even extend to variations in placentation and embryo development.

1.1 Taxonomy

The first true deer, *Dicrocerus* first appears in the fossil record 14.5 million years ago (mya; Eisenberg, 1987). Deer are members of the order Artiodactyla, whose name may be changed to the order Cetartiodactyla now that molecular evidence indicates that whales belong in this group (Gingerich, 2005). The order Artiodactyla also includes pigs, llamas, cattle, sheep, and goats. The time of

emergence of the animals that were the original members of the order is controversial, fossil evidence indicates they appeared around 55 mya but new molecular dating methods suggest that they appeared as far back as 80 mya (Wible *et al*., 2005). The relationship of Artiodactyls to other mammals is similarly uncertain but it appears they shared a common ancestor with Perissodactyls, the order that contains the horse, between 65 mya and 80 mya and that a common ancestor to both Artiodactyla and Primates existed between 70 and 90 mya (Wible *et al*., 2005). Among the order Artiodactyla, the family Cervidae branched off between 23 and 26 mya very close to the time that the family Bovinae appeared (21 – 23 mya) and after the branching of the families Giraffidae (29 mya), Camelidae (37 mya) and Suidae (60 mya) (Hassanin & Douzery, 2003, Lioupis *et al*., 1997; Fig. 1.1).

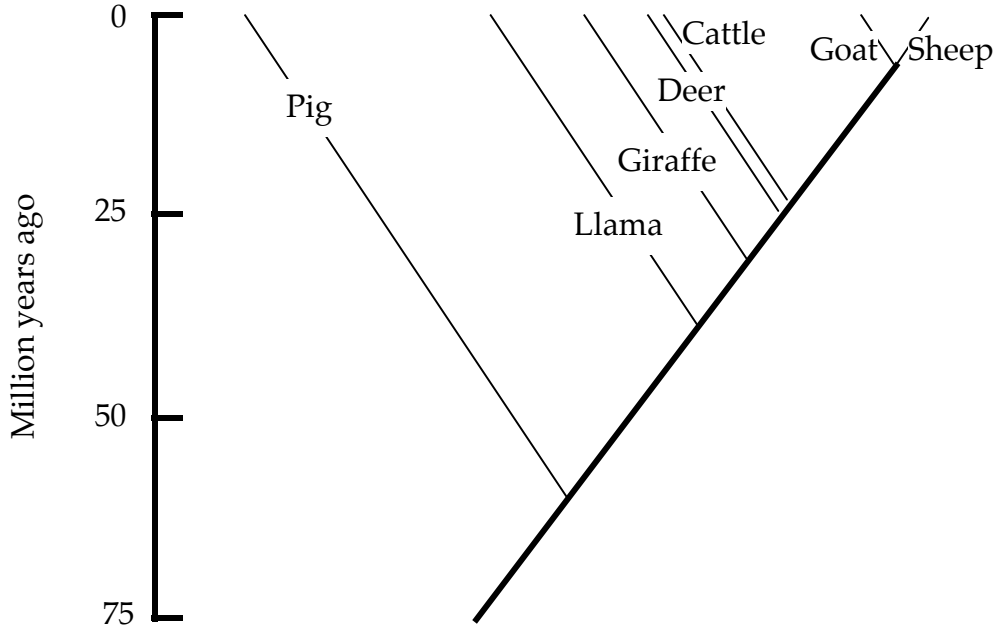

Figure 1.1 Phylogenetic tree for the order Artiodactyla. The tree was constructed with information from Lioupus *et al*., (1997) and Hassanin & Douzery, (2003).

The Cervidae can be subdivided into two groups, the Telemetacarpi and the Pleisometacarpi, based on the degree of regression of the lateral metacarpal bone. The Telemetacarpi are considered the most primitive and are the most diverse with over 10 genera. The Telemetacarpi are also called the New World subfamily or Odocoileinae because many of its members reside in the Americas (Harrington, 1983). The Pleisometacarpi are designated as Old World or Cervinae and contain 6 genera including *Cervus elaphus* (Fig. 1.2).

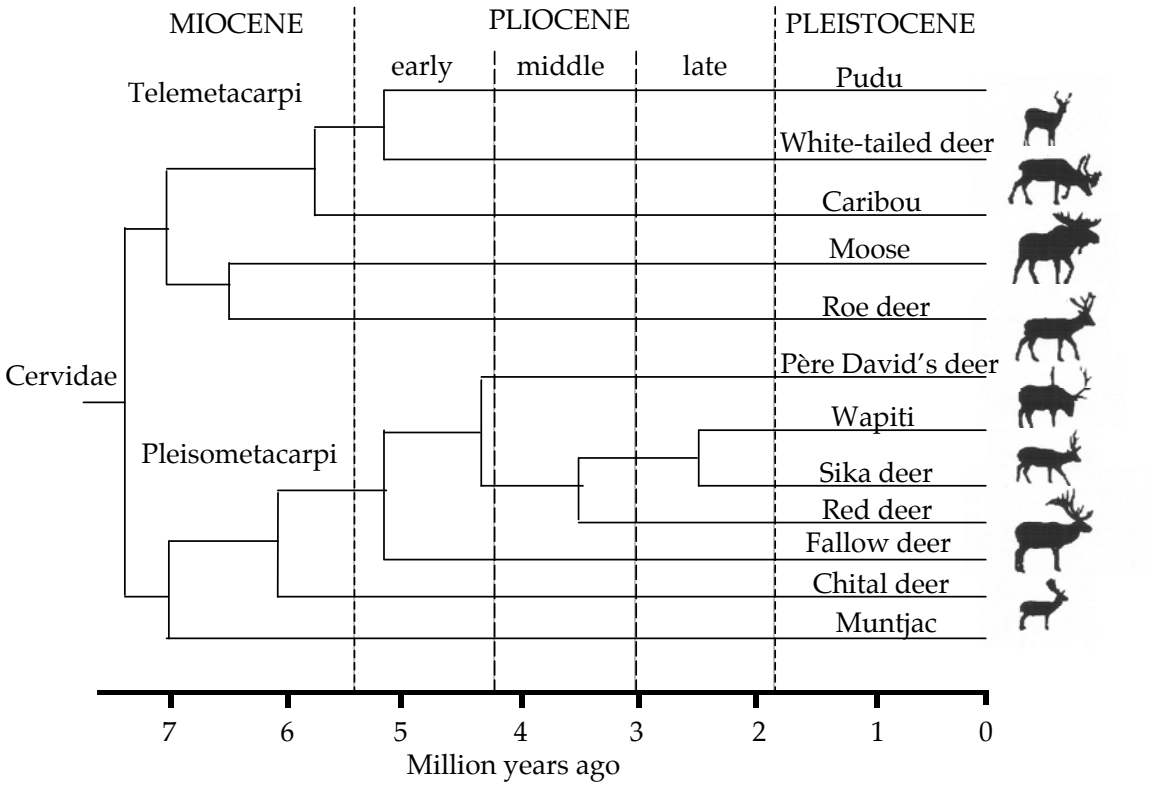

Figure 1.2. Phylogenetic tree for the family Cervidae. The tree was constructed with information from O'Gara (2002) and Pitra *et al* (2004).

1.2 *Cervus elaphus*

Cervus elaphus identifies a species whose natural range can be found in the northern hemisphere reaching in a continuous line from the British Isles on one end all the way to North America on the other extreme (Mahmut *et al*., 2002). Recently an argument has been made to divide the deer known as the North American Elk or wapiti into a separate species. A population of animals is usually regarded as being of the same species if groups within that population will actually or potentially interbreed even though they are reproductively isolated from each other in their normal environment. Fertile crosses of the European red deer and wapiti from North America occur and are commonly produced on game farms in many countries (Haigh & Hudson, 1993). However, recent molecular analysis has identified genetic differences that reflect a long period of isolation and independent development that has resulted in phenotypic and behavioral changes that suggest that the wapiti of North American and western Asia be considered a separate species from the red deer of eastern Asia and Europe (Mahmut *et al*., 2002, Pitra *et al*., 2004, Polziehn & Strobeck, 2002, Randi *et al*., 2001, Schonewal, 1994).

Current evidence of the evolutionary history of *Cervus elaphus* indicates central Asia as the location where the species first developed sometime during the Pliocene (5.2 – 1.8 mya; Mahmut *et al*., 2002). Migrations then followed in both easterly and westerly directions with *C. elaphus* appearing in Europe in the early Pleistocene (0.7 – 0.5 mya; Fig. 1.3; Mahmut *et al*., 2002, O'Gara & Dundas, 2002). The earliest fossil evidence indicates that *C. elaphus* may have arrived in North

America during the late Pliocene or early Pleistocene (~2 mya) but the evidence remains controversial (O'Gara & Dundas, 2002). Most fossil evidence is dated to the Wisconsin glacial stage (90,000 – 10,000 years ago). At this time a land bridge existed between Alaska and Siberia and apparently provided suitable habitat for wapiti (O'Gara, 2002, O'Gara & Dundas, 2002). It is believed that the glacial recession during the last interglacial stage about 10,000 years ago allowed the Alaskan wapiti to spread southward and populate most of North America (Gutherie, 1966). This is substantiated by the rare nature of fossils before 10,000 years ago and the numerous fossils that are present since that time (O'Gara & Dundas, 2002). DNA evidence also suggests that wapiti are recent immigrants to North America. The close relationship between wapiti of the mountains of northern Asia and all North American wapiti mean these populations have not been separated for long (Mahmut *et al*., 2002, Polziehn & Strobeck, 2002) and that if earlier migrations did take place they are not ancestral to the present day North American wapiti.

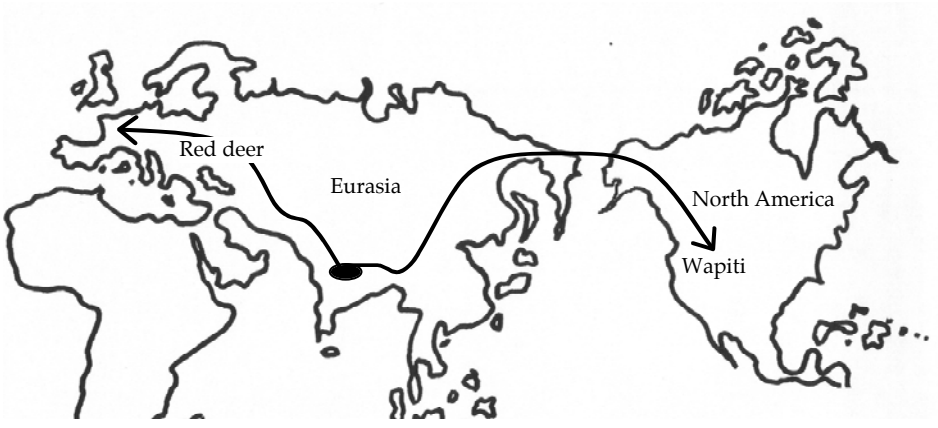

Figure 1.3.The ancestral migrations of wapiti. The black oval designates the location where the ancestor to *Cervus elaphus* was thought to have originated.

The family Cervidae developed in the northern hemisphere in Asia and probably lived in a warm, subtropical climate (Jabbour *et al*., 1997). From that time many species of deer have developed and found their way into various environmental niches. Those species that are found in the temperate and cold zones (30 – 80° latitude) have clear seasonal behaviour and reproduction and those that are found in the tropical and subtropical zones (0 – 30° latitude) may display limited seasonality: annual cycles that are not synchronized between different deer species in the same area or are completely aseasonal (Lincoln, 1983, 1990, Loudon & Brinklow, 1990). Deer species also vary in other aspects, some being monovular, and bearing a single offspring (e.g. fallow deer, *Dama dama*; Asher, 1985), and others polyovular (e.g. white-tailed deer, *Odocoileus virginianus* Verme & Ullrey, 1984) with multiple offspring annually. Variation also exists in placentation and embryo development, with the extreme example being roe deer (*Capreolus capreolus*), which experience embryonic diapause (Aitken, 1974).

Wapiti are a large bodied deer, with stags weighing up to 450 kg and hinds up to 300 kg (Haigh & Hudson, 1993). As with most deer, the stags have boney antlers that are polished and hard during the breeding season and are replaced every year. Two extremes of life-history strategies for which species have been selected have been described. One maximizes the reproductive capacity of individuals and therefore the potential growth of the population, this is referred to as the r-strategy. The other, referred to as the K-strategy, maximizes the competitive ability of individuals, which enhances the stability of the population (Harrington, 1983). The habitat frequented by the species tends to

favour the development of one strategy or the other. Deer of the Plesiometacarpi subfamily tend to be K-stategists and as such they have a lower reproductive potential; females mature at 2 years of age and normally have single births. In addition K-strategists are usually mixed grazers/browsers and can live on diets that are mainly roughage (Harrington, 1983). All of these characteristics apply to wapiti. In comparison the Telemetacarpi are generally r-strategists that have multiple births and early sexual maturation and are usually adapted to feed on concentrates or browse.

1.3 Incentive for research

One of the motivations for studies of reproductive function in deer is the need to support conservation efforts for deer species that are endangered and are being maintained in captive environments. However, most reproductive studies have been initiated in recent years in response to the latest attempts to use deer in agriculture. Husbanding of deer has been recorded several times in human history. Evidence has been found that suggests the husbanding of deer took place as early as 13,000 B.C. and records indicate that the Persians maintained hunting reserves in 1,000 B.C. Roman documents contain many references to game husbandry during the first century B.C. (Haigh & Hudson, 1993). The most modern incarnation of deer farming has its origins in New Zealand. European settlers who wanted a large game species to hunt on the island that was previously free of large ungulates had introduced deer, primarily red deer, to New Zealand. With no predators other than hunters, the deer population rose rapidly and began to degrade the environment and threaten native species

(Deer Industry New Zealand, 2006). In order to protect the environment, the government of New Zealand authorized a large-scale cull of red deer in the 1960's. The deer were hunted and their products, meat and hides, were sold. The sale of deer products created a market that proved inviting to many New Zealand farmers who, in the 1970's began to set up facilities to house and raise deer with the intent of selling into the markets created by the deer cull and modern deer farming was born (Deer Industry New Zealand, 2006).

The agricultural use of deer has since grown into an international industry involving at least 18 countries (Haigh & Hudson, 1993). New Zealand has the largest population of deer on farms in the world. There were about 5,000 farms with 1.7 million deer in 2004. It is estimated that in 2005 New Zealand exported over 27 million kg of venison and that revenue from the export of all deer products will exceed $250 million (NZ; Deer Industry New Zealand, 2006). In Canada, the most recent estimates put the number of game farms at 1,359 and the number of farmed wapiti at 86,744 (Nixdorf, 2005).

As with other types of animal agriculture there has been a desire to control breeding and to apply advanced techniques of assisted reproductive technologies to deer in order to influence production of desirable traits by genetic manipulation. The application of reproductive technologies to wildlife is hindered by the lack of the basic knowledge that is essential for the enhancement and control of reproduction (Pukazhenthi & Wildt, 2004). An increased understanding of deer reproductive function will not only help further the objectives of agriculture, but will also be valuable in the

conservation efforts being applied to deer under threat of extirpation or extinction. Animals that are conditioned to handling provide an opportunity for daily or more frequent examination and sampling. This offers an advantage over less invasive monitoring methods (e.g., population dynamics, behavior, fecal steroid measurement) in that the analysis can be done more regularly, more precisely, and results can be obtained more rapidly (Pukazhenthi & Wildt, 2004). The 2004 IUCN Red List of Threatened Species contains 13 species of Cervidae that are judged to have a high or very high risk of extinction. Many of the species on the IUCN Red List have different behaviours and ecology from red deer, wapiti, and fallow deer, which are the species studied most intensely. Unfortunately they do not represent the complete spectrum of deer reproductive physiology. However, the study of any deer species is valuable in the effort towards conservation because it adds to the knowledge base that must be drawn on when applying assisted reproductive techniques to species which have limited numbers and are threatened.

Captive breeding programs that either maintain a reservoir for the species or supply animals for future re-introduction to the wild may help many of these species. Maintaining captive breeding animals is facilitated through computerized genetic management programs, which select unrelated individuals that are appropriate out-cross candidates to limit the effects of inbreeding and maintain genetic diversity without exceeding the limited space available to hold the animals. In the past, this required that the selected animals be moved from their herd of origin to the location of the designated herd. This was not only difficult and expensive but also created problems with disease

control and testing. Current assisted reproductive technologies provide the possibility of accomplishing the goal of maintaining genetic diversity without moving animals. Further developments in cyropreservation have led to genome or genetic resource banks (Jabbour *et al*., 1997, Pukazhenthi & Wildt, 2004).

These banks can act as an insurance policy against catastrophes. Another and possibly more potent insurance policy is the creation of banks of cryopreserved embryos that contain the full genetic complement of both the sire and the dam. The potential of cryopreserved embryos to help protect and manage a species is great. It would have a large effect on maintaining genetic diversity and the integrity of a captive population (Pukazhenthi & Wildt, 2004).

Assisted reproductive technologies (ART) include techniques like artificial insemination, estrous synchronization, embryo transfer, and in-vitro fertilization. These techniques have been developed and applied to cattle and are now commercially available (Looney *et al*., 1994). They are used in modern agriculture to increase productivity by efficiently distributing desirable genetics over a large population of animals that is widely distributed. Desirable traits are monitored through the use of detailed record keeping and superior individuals are then identified. The genetic material from these individuals can be collected and quickly disseminated throughout the breeding herd leading to an increase in overall productivity that otherwise would not have occurred or would have taken much longer to obtain (Lohuis, 1995).

Assisted reproductive technologies are only a set of tools and increasingly members of the tool set rely on other members of the same tool set in order to be effective. The successful application of embryo transfer relies on the development of estrus synchronization, artificial insemination, and embryo cryopreservation. To increase the likelihood of success it is important to obtain as much knowledge on the reproductive function of the target species as possible before the tools of ART are applied. Most of the knowledge gained to date is for long-domesticated species and is not readily applicable to wildlife or recently domesticated species, therefore it is important to study new species to expand the knowledge base and increase the number of species to which these tools can be applied.

1.4 Reproduction

1.4.1 Reproductive Anatomy

The female reproductive tract in wapiti is similar to that of other ruminants, much as cattle and sheep. The entire reproductive tract is contained within the pelvic cavity when non-pregnant (Fisher & Fennessy, 1985, Glover, 1985). The uterus has two ventrally curled horns, which are 5 – 8 cm in length in the nulliparus hind. The body of the uterus is short, about 3 cm long. There are 3 to 7 caruncles on the mesometrial side of each horn. The cervix is 10 to 15 cm long and narrow, especial in the nulliparus hind (1.5 cm in diameter), and has a complex arrangement of 4 to 6 cervical rings. The vagina is approximately 20 cm long (Fisher & Fennessy, 1985). The ovaries have migrated caudally as in domestic ruminants and are found near the tip of each uterine horn within the

pelvic cavity in the non-pregnant animal. The ovaries are small measuring 15 x 5 x 8 mm (Glover, 1985).

1.4.2 Seasonality

Wapiti live in the temperate and cold zones of the earth and are seasonally polyestrous (Glover, 1985, Lincoln, 1990, Loudon & Brinklow, 1990, Morrison, 1960a). In North America the breeding season begins in late September, most animals becoming pregnant during the period of intense breeding activity, distinguished by aggressive behavior of stags, known as the rut (Guinness *et al.*, 1971, Struhsaker, 1967). The rut is complete by the end of October (Wishart, 1981). *Cervus elaphus* is monotocous, with twins occurring rarely (Guinness et al., 1971, 1978). The average conception date in two herds in North-Western United States was estimated to be the first week of October (Morrison *et al.*, 1959). Hinds that fail to conceive continue to cycle as late as February of the following year (Hudson et al., 2002, Morrison, 1960a). At that time, ovulation ceases until the onset of the next breeding season. Pregnant hinds give birth from late May until early July (Hudson *et al.*, 2002).

1.4.2.1 Retino-pineal pathway

The seasonal behaviour of wapiti is controlled by photoperiod (Lincoln, 1983, Loudon & Brinklow, 1990). The mechanism by which the length of daylight controls the reproductive function has been studied mainly in sheep, which are seasonal, short-day breeders (Karsch *et al*., 1984). Sheep therefore will be used as an example and known differences with deer will be discussed later. Sheep determine the length of the day from the length of uninterrupted darkness (Aleandri *et al*., 1996, Dunlap *et al*., 2004). When the eye detects light, nervous impulses travel a route called the retino-pineal pathway (Fig. 1.4). Retinal photoreceptors detect light and impulses are sent to the superchiasmatic nucleus (SCN). From there the impulse is transmitted by other nerves to the paraventricular nucleus and then to the intermediolateral cell column of the spinal cord. Nerve fibres from the spinal cord travel to the superior cervical ganglion via the sympathetic trunk. The nervous signal is then transmitted to the pineal body located on the roof of the forebrain near the third ventricle via the sympathetic noradrenergenic fibres (Binkley, 1993, Dunlap *et al*., 2004, Reiter, 1983). The pineal gland is the only endocrine gland that is directly influenced by the external environment. The pineal gland converts environmental signals from the retina into endocrine messages (Binkley, 1993, Dunlap *et al*., 2004, Reiter, 1983).

The noradrenergic fibres stimulate α and β adrenergic receptors on the pinealocytes and cause the release of cAMP and cGMP. An increase in cAMP causes an increase in N-acetyltransferase (NAT) activity. N-acetyltransferase is

the rate-limiting enzyme in the production of melatonin from serotonin. N-acetyltransferase converts serotonin to N-acetylserotonin, which is then acted on by hydroxy indole-O-methyltransferase (HIOMT) to become melatonin (Aleandri *et al*., 1996). Melatonin is the major product of the pineal gland and the hormone that is used by the body to monitor day length. Melatonin is not stored, instead it is immediately secreted into the peripheral blood (Dunlap *et al*., 2004) and the cerebrospinal fluid where the highest concentrations are found (Malpaux *et al*., 2001). Melatonin is lipophylic and this property ensures that all tissues receive an endocrine signal of time of day based on melatonin concentration and time of year based on the duration of increased melatonin concentration (Lincoln *et al*., 2003, Reiter, 1991).

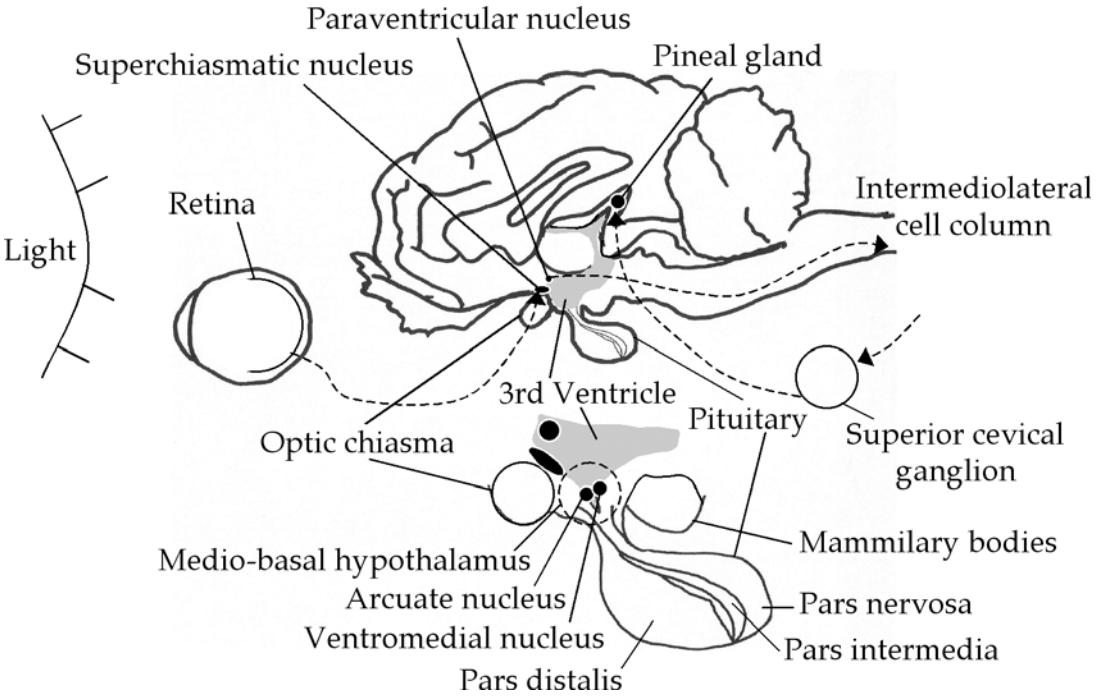

Figure 1.4. Retino-pineal pathway. This pathway conveys information on day length to the pineal gland, which then converts it to a neuroendocrine message. In sheep circadian timer cells are found in the superchiasmatic nucleus and calendar cells are found in the medio-basa hypothalamus (Aleandri *et al*., 1996, Malpaux *et al*., 1998).

Exposing the retina to light sends a signal that interrupts the production of melatonin by reducing the activity of NAT. The interruption of melatonin production occurs regardless of the time of day the stimulus is received (Dunlap *et al*., 2004, Lincoln *et al*., 2003). Serum melatonin concentrations peak during darkness and the response to photoperiod is dependent on how the duration of the melatonin signal is decoded in melatonin-responsive tissues (Dunlap *et al*., 2004, Lincoln *et al*., 2003). As day length increases the duration and concentration of melatonin decreases.

Circadian timing is generated autonomously by a small number of clock genes that interact to control their own transcription. Clock genes are expressed in the SCN and provide the basis for circadian pacemaker functions (Dunlap *et al*., 2004, Lincoln *et al*., 2003)}. The SCN circadian clock is set by the periodic stimulation with light every 24 hours and regulates the diurnal rhythms of activity and sleep, body temperature, pituitary activity, and the nocturnal release of melatonin (Lincoln *et al*., 2003). The circadian rhythm combined with an overriding response to light that suspends melatonin production ensures that melatonin is only produced at night and the duration and hence quantity of melatonin release depends on the uninterrupted length of night (Dunlap *et al*., 2004).

1.4.2.2 Circannual rhythm

At the base of seasonal reproduction is an innate mechanism called the circannual rhythm. This circannual rhythm serves to coordinate the physiological changes necessary to cause the initiation of ovulation and the maintenance of regular estrous cycles. The length of daylight is used by the animal as a reference to reset the timing of the circannual rhythm so that the breeding season occurs at the appropriate time of the year to allow the birth of offspring when the environment is most favorable to their survival (Dunlap *et al*., 2004, Gwinner, 1986).

The pattern of melatonin production by the pineal gland is the signal that entrains the circannual rhythm and ensures that the rhythm coincides with other individuals in the group and is aligned with the seasons (Dunlap *et al*., 2004, Gwinner, 1986)}. If the pineal gland is removed the circannual rhythm will run free and will no longer be necessarily coordinated with others in the group or the season (Woodfill et al., 1994). When the circannual rhythm is left to run free, the reproductive system of the animal will pass through the regular seasonal changes but they will occur in less than 12 months. For example in sika deer (*Cervus nippon*), the free running circannual rhythm is 9 to 10 months (Gwinner, 1986). Innate circannual rhythms have several advantages for seasonal species. They act as buffer against environmental variation and prevent the animal's physiology from misidentifying the season thereby providing consistency between years (Gwinner, 1986). This is very important for species in a temperate climate. Their life cycle must be precisely timed in

order to ensure survival and local weather conditions cannot be allowed to interfere. It is also essential for migratory species that over-winter in areas which are seasonally constant and provide few clues as to the time of year to start migration (Gwinner, 1986).

In sheep, the circadian pattern of melatonin production that is most effective at synchronizing or entraining the circannual rhythm is the pattern created during the spring and summer (Barrell *et al*., 2000). A circadian pattern of melatonin on its own is not enough to entrain the circannual rhythm it must be the pattern created during the spring and summer around the summer solstice (long day). However, the pattern needs only to be present for as few as 70 days a year to maintain the entrainment of the circannual rhythm (Dunlap *et al*., 2004, Woodfill *et al*., 1991), which begins the process that will result in ovulations resuming in the fall. Ovulation will resume at a set time after melatonin concentrations stop declining. The decreased day length of autumn is not needed for the initiation of the breeding season. Indeed, an early decrease in the photoperiod after the summer solstice or increasing the photoperiod as the summer solstice approaches does not affect the onset of the next breeding season (Deveson *et al*., 1992). The autumn pattern of melatonin production (short days) is needed to maintain the full duration of the breeding season (Barrell *et al*., 2000) and is also necessary for the long day pattern to be recognized (Malpaux & Karsch, 1990).

1.4.2.3 Effect on the hypothalamo-pituitary axis

When the short day pattern of melatonin production is present there is an increase in the pulse frequency of luteinizing hormone (LH) and an increase in gonadotropin releasing hormone (GnRH) secretion. Conversely, when a long day pattern of melatonin production is present there is a decrease in LH release (Viguie *et al*., 1995). However, melatonin does not have a direct effect on the pituitary or on the gonad. Instead its primary effect is mediated through pituitary gonadotrophic hormones (Goldman, 1999). The influence of photoperiod on the pulsitile secretion of LH is mediated by melatonin through an increased sensitivity of the hypothalamic-pituitary axis to the negative feedback of gonadal steroid (estradiol in females) hormones (Dunlap *et al*., 2004, Woodfill *et al*., 1994)}.

The binding sites for melatonin in sheep are found in the SCN and the medio-basal hypothalamus (Malpaux *et al*., 1993), but the binding sites with highest affinity are found in the pars tuberalis, which have seasonal and species-specific variations (Pevet, 2003). However, the melatonin binding sites at the pars tuberalis are more involved with prolactin regulation and strong evidence does not exist that indicates binding sites at this location affect reproduction in sheep (Kennaway & Rowe, 1995). The ventromedial and arcuate nuclei of the medio-basal hypothalamus bind melatonin and affect reproduction in sheep (Malpaux *et al*., 1998, Malpaux *et al*., 1996).

Melatonin sensitive calendar cells reside in the ventromedial nucleus located in the medio-basal hypothalamus (Malpaux *et al*., 1993, Malpaux *et al*., 1998). Calendar cells detect the duration of increased melatonin concentration. These cells enable the seasonal control of the gonadotrophin/gonadal axis (Thiery *et al*., 2002). However, the majority of GnRH secreting neurons (60%) are located in the pre-optic area of the brain and therefore interneurons are required to communicate between the melatonin responsive calendar cells of the medio-basal hypothalamus and the GnRH secreting cells.

The neural pathway between the melatonin responsive calendar cells and the GnRH secreting cells located in the hypothalamus is thyroxine dependent (Billings *et al*., 2002). Thyroid hormones are required for transition to the anestrus season, but thyroid hormones are only required to be present for a critical period of 60 to 70 days near the end of the breeding season (Dunlap *et al*., 2004, Thrun *et al*., 1997). Experimental evidence indicates the process that leads to the cessation of ovulation takes weeks to develop. The time it takes probably indicates the complexity of the neural changes that are being made (Malpaux *et al*., 1996, Thrun *et al*., 1997, Viguie *et al*., 1997).

The effect of the thyroxine dependent neural pathway between the calendar cells and the GnRH secreting cells is manifest by the enhanced negative feedback effect of estradiol on GnRH secretion. During the transition to anestrus the pulse frequencies of GnRH and LH secretion decrease from 10 pulses every 6 hours under short day exposure to 1 pulse every 6 hours under long day exposure (Viguie *et al*., 1995). Once the enhanced negative feedback of estradiol

has taken effect, LH secretion falls to an undetectable level (Billings *et al*., 2002). This pronounced negative feedback effect of estradiol on the release of GnRH is what finally brings about the anestrous season. Interestingly prolactin, another hormone seasonally regulated by melatonin production and is important in hair growth, is not affected by the absence of thyroid hormones. Prolactin and LH are regulated by melatonin at different sites (Billings *et al*., 2002).

In sheep the calendar cells also communicate via thyroxine dependent pathways with dopaminergic neurons that in turn communicate with the median eminence of the pituitary (Viguie *et al*., 1995, 1997) where the GnRH secreting cells have their terminus. The dopaminergic cells inhibit GnRH release at the level of the median eminence. Dopamine concentrations are increased when melatonin concentrations are decreased (Malpaux *et al*., 1997). Therefore during long days dopamine concentrations would be elevated. Additionally, estradiol treatment induces the enzyme tyrosine hydralase (TH), which is the rate limiting enzyme in dopamine production and short days inhibit TH activity at the median eminence of the pituitary (Malpaux *et al*., 1997, Viguie *et al*., 1997). Therefore, photoperiodic regulation of LH secretion is accomplished in part by a melatonin-dependent modulation of the activity of dopaminergic inhibitory inputs on the GnRH terminals in the median eminence and by the enhanced negative feedback of estradiol on GnRH secreting cells.

1.4.2.4 Differences found in deer

The seasonal mechanism in deer is thought to be very similar to that found in sheep but several differences have been reported suggesting that other mechanisms are also at work controlling seasonality. Melatonin receptors are found more widely distributed in the brain (Williams & Helliwell, 1993) and are also found in the pars distalis of the pituitary (Williams *et al*., 1996). No melatonin receptors are found in the pituitary of sheep. There is evidence of both steroid-dependent and independent pathways that suppress LH (Anderson & Barrell, 1998, Meikle & Fisher, 1996). However, the steroid-independent pathways do not use dopamine or endogenous opioids for seasonal regulation (Anderson & Barrell, 1998) suggesting a neural mechanism that is different from that of sheep. These steroid-independent pathways are also thought to be more important and that they are larger contributors than in the ewe. The LH pulse frequency is even lower in anestrus than in the ewe and there is a greater difference between seasons (Anderson & Barrell, 1998). Thyroid hormones are required in deer to initiate anestrous (Anderson & Barrell, 1998, Billings *et al*., 2002) but suppression of LH still happens even without thyroid hormones. It has been suggested that the pathways are additive (Anderson et al., 2002) and different depths of anestrous control exist (Meikle & Fisher, 1996), which is evident from the reduced response to exogenous GnRH early in anestrous.

1.4.3 Ovarian follicle dynamics

1.4.3.1 Ovarian follicle dynamics in cattle

The wave pattern of follicle development has been studied extensively in cattle. The general pattern of follicle development in cattle, critically observed by transrectal ultrasonography, has been used to define a model for other species (G.P. Adams & Pierson, 1995). An essential requirement for characterizing follicle development is the ability to monitor individually identified follicles. This was found to be possible in cattle using ultrasonography (Pierson & Ginther, 1987b, Pierson & Ginther, 1988). The technique of characterizing follicle development with transrectal ultrasonography involved detailed sketches that were made of both ovaries on a daily basis noting the size and location of follicles and corpora lutea. This was facilitated by the tendency of the ovary to maintain a constant orientation from day to day in the cow. From retrospective examination of serial ovarian sketches, it was possible to identify and trace the development of individual follicles and a direct test of the 2-wave theory of follicle development in cattle was made in 1989 (Knopf *et al*., 1989).

At the time of ovulation, follicle development begins with the simultaneous growth of a group of antral follicles, a wave. The wave is first ultrasonographically detected as growing follicles reach a diameter of 3 to 4 mm, this point is termed "wave emergence" (Ginther *et al*., 1989a, 1989b, 1996, Pierson & Ginther, 1987a, Savio *et al*., 1988, Sirois & Fortune, 1988). This group of follicles continues to grow for the next 2 or 3 days at which point one follicle, referred to as the dominant follicle, becomes distinctly larger than the rest of the

cohort and continues to grow while the remaining follicles cease growing and begin to regress (Ginther *et al*., 1996). Regular, periodic nonovulatory follicular waves occur during the estrous cycle until luteal regression occurs. The dominant follicle present when the corpus luteum regresses continues to develop and then ovulates. Most estrous cycles in cattle are made up of 2 or 3 waves of follicle development (Ginther *et al*., 1996).

The emergence of a wave of follicles has been associated temporally with a surge in follicle stimulating hormone (FSH; Adams *et al*., 1992a)). Cows with 2 waves have 2 surges in FSH and cows with 3 waves of follicle development have 3 surges in FSH. The peak in FSH occurs at the time that the follicle of the wave destined to become the dominant follicle reaches 4 mm in diameter. Thus, the surge in FSH precedes the detection of wave emergence. The decline in FSH occurs when the future dominant follicle is 6 mm in diameter. At this stage in development, it is known that the follicle is capable of producing estradiol and inhibin (Ginther *et al*., 2001). The production of estradiol and inhibin by the cohort of developing follicles is a possible explanation for the suppression of FSH production. Exogenous estrogens and the proteinaceous components of follicular fluid are known to have a negative effect on FSH production (Bo *et al*., 1993, Kastelic *et al*., 1990). The decline in FSH is associated with selection of the dominant follicle (Adams *et al*., 1993a, 1993b). Once selected, the dominant follicle continues to grow despite declining FSH levels, whereas the remaining follicles of the wave cease their growth and begin to regress. The dominant follicle is able to continue its growth by changing its gonadotropin dependency from FSH to LH (Ginther *et al*., 2001). The subordinate follicles do not acquire

the ability to use to LH and are deprived of FSH required for their survival. LH has been shown to be necessary for follicles to develop past 7 to 9 mm in diameter in cattle (Gong *et al*., 1995).

Dominant follicles of nonovulatory waves develop in the presence of progesterone, which suppresses LH levels (Adams *et al*., 1992a). The dominant follicle of a nonovulatory wave may participate in its own demise by suppressing FSH to basal levels in an environment with LH levels that are not high enough to allow continued growth and survival. The dominant follicle ceases its growth and soon thereafter begins to reduce its production of estradiol and inhibin. This drop in the products required for the negative feedback of FSH leads to a surge in FSH that stimulates the emergence of the next wave of follicle development (Adams *et al*., 1992b). If the corpus luteum regresses while the dominant follicle is growing, the resulting drop in progesterone allows for an increase in LH pulse frequency. The increase in LH pulse frequency stimulates estradiol production in the dominant follicle. The drop in progesterone followed by the increase in estradiol induces the estrous behaviours normally associated with ovulation. High levels of estradiol secretion in a low progesterone environment have a positive feedback effect on LH secretion, which in turn stimulates further estradiol production from the dominant follicle. Estradiol levels continue to increase until they stimulate a large surge in LH release. The pre-ovulatory LH surge causes a series of changes within the dominant follicle that result in ovulation (Espey & Lipner, 1994). An analogous pattern of estrous cycle follicle development has been observed in other ruminants, namely sheep, goats, llamas and muskoxen that

have been similarly studied (Adams *et al*., 1990, Ginther & Kot, 1994, Hoare *et al*., 1997, Ravindra *et al*., 1994).

1.4.3.2 Ovarian follicle dynamics in deer

Ovarian follicle development during the estrous season has been studied in Columbian black-tailed deer using gross and histologic analysis of the ovaries and reproductive tracts gathered from 444 hunter-killed animals (Thomas & Cowan, 1975). Cycles of follicle development were described. After ovulation, only the smallest antral follicles were found not to be atretic and a new generation of follicles grew rapidly over the next 5 days to preovulatory size. This group of follicles then became highly vascularized and began to degenerate about 8 to 10 days after ovulation. These follicles were then replaced by another cycle of follicle development. A small, short-lived CL followed the first ovulation of the season. The second ovulation occurred 6 to 11 days later. Behavioural estrus was noted at the second ovulation of the season but was not always associated with the first ovulation. In the nonpregnant animal, it was suggested that the follicle cycles would continue until the end of the functional life of the CL at which point the follicles would not regress but would ovulate. Estrous behaviour occurred every 23 to 29 days; therefore 2 or 3 cycles of follicle development presumably took place. The follicle cycles described by Thomas and Cowan (1975) bare great similarity to the waves of follicle development described in cattle and it is probable that they would be called waves today.

Ovarian follicle development in red deer, a subspecies of *Cervus elaphus,* has been examined by the dissection of collected ovaries (McLeod *et al*., 1996). This was done at known times before ovulation and at the mid-luteal phase of the estrous cycle during the breeding season. A wide variation in the number of follicles was found between individuals. Irrespective of the time of collection, there was at least one large (7.5 mm in diameter) non-atretic follicle present within the pair of ovaries, but no obvious pattern of preovulatory follicle development during the follicular phase of the estrous cycle was found. The difficulty in finding a pattern of preovulatory follicle development probably lies in the variability between individuals; the limited number of days of the observation period; and the inability to observe follicle development daily in the same ovary.

Transvaginal ultrasonography has been used to characterize antral follicle development in red deer (Asher *et al*., 1997). Surgically modified hinds (ovaries adherent to vaginal wall to facilitate locating them daily) were examined during the normal estrous cycle. The total number of follicles ≥ 3 mm did not vary during the estrous cycle. The mean follicular size did vary with the largest follicle diameters being most common on days 8 to 10 and days 15 to 18 (day 0 = day of ovulation). The smallest mean follicle size was recorded on day 1. When the number of large follicles (≥ 6 mm) was examined for one complete estrous cycle, three possible patterns were seen: one large follicle over the complete cycle, two consecutive large follicles, or three consecutive large follicles. New follicles were seen to emerge upon the regression or ovulation of the large follicles. However, Asher *et al.* (1997) concluded that no evidence was found to

support the regular, periodic appearance of waves of follicle development during the estrous season, but rather the appearance of irregular waves of follicle development.

At the onset of the estrous season, interovulatory intervals of 7 to 9 days duration were detected in red deer (Asher *et al*., 1997). A peak in follicle number characterized these cycles on days 4 to 5 and the mean maximum follicle diameter occurred on days 6 to 7 (day 0 = day of ovulation). Each short cycle that was identified was followed by an estrous cycle, which averaged 18 days in length.

Ovarian function in fallow deer (*Dama dama*) has been studied using transrectal ultrasonography to characterize follicle dynamics during the estrous season (Asher *et al*., 1999). A temporal pattern of the appearance of a single large (≥ 6 mm) follicle associated with the disappearance of small follicles was interpreted as a dominant follicle wave. Two or three waves were common during one estrous cycle. The greatest mean number of small follicles was noted on days 1 to 3 and the greatest mean number of large follicles was found on days 8 to 9 and 20 to 22 of a 22-day estrous cycle (day 0 = day of estrus). This report provides evidence of regular follicle waves in deer similar to those described in cattle and sheep. The transition from the anestrous to estrous season was characterized by a short first estrous cycle, which was half the length of a normal estrous cycle and the transition into the anestrous season was characterized by an increase in length of the estrous cycle and estrous cycles that became more irregular as the anestrous season approached (Asher, 1985).

1.4.3.3 Ovarian follicle dynamics in wapiti

Ovarian function has been studied by examining ovaries harvested from wapiti hinds of known breeding history and by rectal palpation (Glover, 1985, Morrison, 1960b). The estrous cycle in wapiti is 20 days in length (Glover, 1985) and short estrous cycles have been recorded during the transition from the anestrous season to the estrous season (Morrison, 1960a). However, ovarian follicle dynamics have not been well described in wapiti. Glover's (1985) findings indicated that wapiti may have a 6 to 7 day cycle of follicular development during the estrous cycle and that follicle development was not found in the late luteal phase (day 13 to 15) but resumed after luteal regression.

The seasonal transitions from anestrus to estrus and estrus to anestrus were studied using serial transrectal palpation in wapiti (Glover, 1985), the author concluded that this time period was marked by irregular follicle development leading to estrous cycles of varying lengths that may or may not include ovulation. The author also reported that irregular circulating concentrations of progesterone that were only slightly elevated above detectable levels characterized the transition. At the end of the breeding season in mid- to late-winter, the transition into the anestrous season occurred over a longer time period (2 months) than the transition into the estrus season (Glover, 1985, Guinness *et al*., 1971) and the estrous cycle was reported to increase in length and become more irregular as the anestrous season approached (Glover, 1985).

A recent study in wapiti using transrectal ultrasonography during the anestrous season provided evidence that antral follicle development occured in a wave-like pattern (McCorkell et al., 2004). The regular appearance of peaks and troughs in follicle numbers was readily apparent and the interval between one peak in follicle numbers to the next was the same as the interval between troughs in follicle numbers (6.8 ± 0.4 days). These intervals were very similar to the interval between the emergence of successive large follicles, which was 7.1 ± 0.5 days. The similarity of the intervals between peaks, troughs and large follicle emergence was consistent with a wave pattern of follicle development and led to the conclusion that a wave of follicle development occured about every 7 days during the anovulatory season in wapiti (Fig. 1.5). In addition, the temporal association between the emergence of the largest follicle and a decrease in follicle numbers was consistent with the phenomenon of follicular dominance, similar to that described in cattle (Ko *et al*., 1991), suggesting that the largest follicle was functionally dominant. Selection of the follicle destined to become the dominant follicle was manifest within 1 day of follicle wave emergence. The dominant follicle grew to a much larger diameter and had a significantly longer life span than other follicles emerging at the same time. In addition, 1 day after the dominant follicle stopped growing, a new wave of follicle development began to grow, indicating that the dominant influence was lost shortly after the dominant follicle stopped growing.

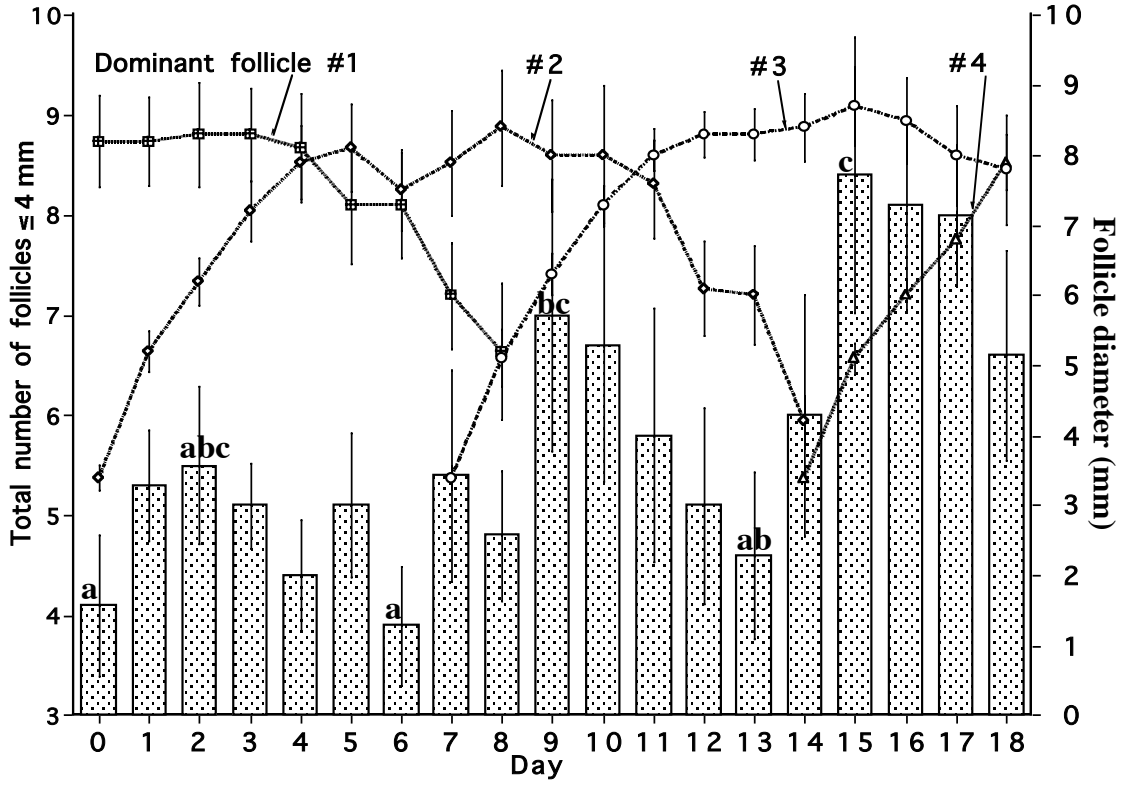

Figure 1.5. Profiles of the diameter of successive dominant follicles (lines) and the number of follicles (≥ 4 mm) detected on each day (bars) during the anestrous season (McCorkell *et al.*, 2004). Values without common superscript are different ($P < 0.05$).

1.4.4 Exogenous control of ovarian function in deer

1.4.4.1 Melatonin

Translocating deer to the southern hemisphere results in the shifting of the breeding season by 6 months so that it coincides with the light cues of the southern hemisphere (Fletcher, 1974). This process does not occur immediately but over the period of several months all animals will finally adapt to the change. Manipulating the photoperiod is then one way to exogenously control the breeding season of wapiti. Another method is to administer melatonin,

which is the hormone the body uses to monitor photoperiod. Melatonin has been used to advance the breeding season in New Zealand and Australia where the prime grazing periods do not align with the naturally produced calving periods (Adam *et al*., 1989, Asher *et al*., 1996, 1993b, Fisher & Fennessy, 1987). Melatonin is administered to mimic the naturally occurring melatonin concentrations of the short days of autumn by oral, intramuscular and subcutaneous routes and has been found to advance the breeding season by 30 days (Fisher & Fennessy, 1987).

1.4.4.2 Stag effect

The "stag effect" has also been used to synchronize breeding in red deer. Similar to the "ram effect" in sheep the presence of a vasectomized rutting stag induces hinds to enter into the breeding season and therefore more hinds are ready to breed when the selected stag is introduced into the breeding group (Fisher & Fennessy, 1987). Treating the stags alone with melatonin to get them to enter the rut early will subsequently induce the hinds to begin to cycle and is another technique used to advance the breeding season (Asher *et al*., 1996)

1.4.4.3 Estrous synchronization

Estrous detection is difficult in deer because there are not many signs indicating impending estrus that can be observed (Morrison, 1960a). The use of estrous synchronization techniques over comes this problem and an empirically designed treatment protocol to synchronize estrus has been used to facilitate

artificial insemination in red deer and wapiti (Fennessy *et al.*, 1989). The synchronization protocol was first developed in sheep (Robinson, 1965) on the assumption that luteal phase progesterone prevents final follicular maturation in an otherwise continuous pattern of follicle growth; i.e., that an ovulatory follicle maybe selected from an ever-ready pool based on the coincidence of its maturity and the onset of luteolysis (Hafez, 1980).

The estrous synchronization protocol involves the use of a controlled intravaginal drug-releasing device (CIDR), that delivers a continuous concentration of progesterone, and equine chorionic gonadotropin (eCG) upon CIDR withdrawal (Fennessy et al., 1989). The CIDR device is most commonly inserted for 12 to14 days (Bowen, 1989, Fennessy *et al.*, 1990, Fisher *et al.*, 1986). Longer insertion periods have been associated with lower conception rates in red deer (Fennessy *et al.*, 1990). It has become routine to administer 200 to 250 IU of eCG at or near the time of CIDR withdrawal (Fennessy *et al.*, 1989). Three reasons have been cited for this practice (Asher *et al.*, 1993a): 1) an increased incidence of ovulation than with progesterone treatment alone (Fennessy *et al.*, 1989, Fisher *et al.*, 1986), 2) extra gonadotrophic stimulation may be necessary to overcome the effects of stress (Fennessy *et al.*, 1989), and 3) eCG treatment may increase the synchrony of ovulation in a group of hinds (Fennessy *et al.*, 1989). Pregnancy rates in wapiti averaging around 70% have been reported with this protocol (DeGrofft, 2000).

1.4.4.4 Embryo transfer

Embryo transfer, unlike the successful application of AI, has been reported as less successful in wapiti (DeGrofft, 2000, Wenkoff & Bringans, 1991). However, embryo transfer is reported to be about as successful in red deer as in cattle with 3 pregnancies per superstimulated donor female (Fennessy *et al*., 1994). The traditional method of superstimulation in red deer begins with the placement of a CIDR device intravaginally for 12 days. FSH injections are then given twice-daily beginning on the eighth day after CIDR placement and ending 12 to 24 hours after CIDR removal (Fennessy *et al*., 1994). A common addition to the treatment protocol is eCG, which is included to reduce the variability in response (Asher *et al*., 1995, Fennessy *et al*., 1989, Scott *et al*., 2000). It is commonly given in conjunction with the last FSH treatment but improved results have been observed when it was given with the first treatment of FSH (Berg *et al*., 1995).

1.4.4.5 In-vitro fertilization

In-vitro fertilization has been reported in red deer and wapiti (Berg *et al*., 2002, Pollard *et al*., 1995). Oocytes in these studies were obtained from ovaries recovered at slaughter. However, recent work has been done using oocytes recovered by ultrasound guided follicle aspiration in red deer and wapiti (Berg *et al*., 2003). The ability to harvest oocytes repeatedly from a donor hind allows for the production of large numbers of embryos with control of both the sire and dam genetics, which is unlike slaughter-derived oocytes whose exact origin

is not usually preserved in the process of recovering the ovaries and is only a one-time procedure. Superstimulation of follicle development can be used to increase the efficiency of oocyte recovery when repeated collections by transvaginal aspiration are contemplated (Berg *et al.*, 2003).

1.5 Objective

The ultimate goal is to be able to exogenously manipulate ovarian function in order to coordinate natural or artificial breeding or to be able to harvest gametes and embryos in a practical manner. Attaining that goal will make it possible to satisfy the demands that are now coming from the agricultural sector and wildlife conservation. From the conservation point of view, wapiti are members of a large deer genus, Cervus and it is likely that many other members of that genus will share a similar reproductive physiology. It is also likely that all members of the deer family will benefit from a more detailed understanding of the reproductive physiology of one of its members. Advances in cattle reproductive physiology have benefited other artiodactyls. Assisted reproductive techniques that were developed in cattle have found success in the gaur, water buffalo, Armenian red sheep, and llama (Pope & Loskitoff, 1999). From the agricultural point of view, ART is already being applied with some success to red deer, but surprisingly is not as effective in wapiti, even though these animals are considered to be the same species. A better understanding of the reproductive physiology of wapiti may help to uncover the reasons for this apparent difference.

Finally, the general advance in technology has made it possible to observe reproductive function in a new way. Transrectal ultrasonography permits the serial observation of the same ovary in the intact live animal, a feat previously impossible. This advance has led to the refinement and confirmation of the wave theory of follicle development in cattle. Understanding this pattern of follicle development in cattle has led to the development of many novel methods of exogenous control of reproductive function and revolutionized ART in cattle (Adams, 1994). Previously unattainable objectives in cattle like timed-AI and embryo transfer by appointment are now a reality (Bo et al., 2002, Martinez et al., 2000).

The principal hypothesis that guided the studies found in the following chapters was that it was necessary to characterize ovarian function in wapiti during the seasons of the annual reproductive cycle and from that novel methods of exogenous control of ovarian function would result.

To satisfy the hypothesis two objectives were identified. The first objective was to augment the knowledge about endogenous control of ovarian function in wapiti. The second objective was to evaluate novel methods of exogenous control of ovarian function that were based on knowledge gained from satisfying the first objective.

To fulfill the first objective a series of studies were planned to examine ovarian function throughout the annual cycle. The anestrous season has already been studied and therefore the first study involved the estrous season of wapiti. The

second was on the transition from anestrous to the estrous season and the third the transition from the estrous season to the anestrous season. The fourth study was more experimental and involved the present method of exogenous control of ovarian function.

To satisfy the second objective two studies were planned to examine novel methods of exogenous control of ovarian function in wapiti. The first study examined methods of controlling ovarian follicle development and the second study examined new approaches to superstimulate ovarian follicle development.

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2.0 OVARIAN FOLLICULAR AND LUTEAL DYNAMICS IN WAPITI DURING THE ESTROUS CYCLE

2.1 Abstract

The reproductive tracts of 13 mature hinds were examined daily by transrectal ultrasonography and blood samples were taken daily between October and January to characterize follicular, luteal, and endocrine dynamics in wapiti during the estrous season. Follicle development occurred in waves characterized by regular, synchronous development of a group of follicles in temporal succession to a surge in serum FSH concentration. The mean interovulatory interval was 21.3 ± 0.1 days, but was shorter in hinds exhibiting two follicular waves than in hinds exhibiting three or four waves ($P < 0.05$). The interwave interval was similar among waves in two-wave cycles and the first wave of three-wave cycles. All other interwave intervals in three- and four-wave cycles were shorter ($P < 0.05$). The maximum diameter of the dominant follicle of the first wave was similar among two-, three- and four-wave cycles. For all other waves in three- and four-wave cycles, the maximum diameter of the dominant follicle was smaller ($P < 0.05$). Corpus luteum diameter and plasma progesterone concentrations were similar between two- and three-wave cycles, but the luteal phase was longer ($P < 0.05$) in four-wave cycles. The dominant follicle emerged at a diameter of 4 mm at 0.4 ± 0.1 and 0.8 ± 0.1 days

before the largest and second largest subordinate follicles, respectively. The follicle destined to become dominant was larger ($P < 0.05$) than the largest subordinate follicle one day after emergence, which coincided with the first significant decrease in serum FSH concentration. We conclude that the estrous cycle in wapiti is characterized by two, three, or four waves of follicular development each preceded by a surge in circulating FSH, that there is a positive relationship between the number of waves and the duration of the cycle, and an inverse relationship between the number of waves and the magnitude of follicular dominance (diameter and duration of the dominant follicle).

2.2 Introduction

Wapiti are one of 47 existing species of deer and are, along with red deer, a subspecies of *Cervus elaphus*. They are included in the 15 species of deer that live primarily in temperate and cold zones of the earth (Lincoln, 1992). Deer that live in these regions exhibit seasonal changes in their reproductive pattern. Wapiti are seasonally polyestrus (Glover, 1985, Lincoln, 1992, Morrison, 1960a) with recurrent estrous cycles of 21 days, which continue late into winter when they enter anestrus (Morrison, 1960a). *Cervus elaphus* are monotocous, with twins occurring rarely (Guinness *et al.*, 1971, Guinness *et al.*, 1978).

Wapiti are important as game animals, and recently, as a farmed species along with red deer, fallow deer (*Dama dama*), and white-tailed deer (*Odocoileus virginianus*). Interest in the use of assisted reproductive technologies in wapiti has increased for the purpose of propagating genetically valuable animals in farmed or game ranch populations. In modern zoos there is a desire to introduce new genetic vigour into captive cervid populations and obtain offspring from individuals identified through computerized genetic management programs to be unrelated and therefore appropriate out-cross candidates. Artificial insemination has been used quite successfully in red deer and wapiti (Asher *et al.*, 1993, Wenkoff, 2000), but ovarian superstimulation for the purposes of embryo production has apparently not been as successful in wapiti (DeGrofft, 2000). Current technology offers the possibility of creating embryo banks, which have the advantage over frozen semen banks of having the complete genetic complement of both the sire and dam. This would provide

a powerful tool in the protection of threatened species and in the maintenance of genetic heterosis of isolated populations. Currently there are no large-scale embryo banks for any endangered species (Pukazhenthi & Wildt, 2004).

The application of reproductive technologies in wildlife species is hindered by the lack of basic knowledge that is essential for the enhancement and control of reproduction (Pukazhenthi & Wildt, 2004). Animals that are conditioned to handling provide the opportunity for daily or more frequent examination and sampling, which offers the advantage over less invasive monitoring methods (e.g., population dynamics, behavior, fecal steroid measurement) in that the analysis can be done more regularly, more precisely, and results can be obtained more rapidly (Pukazhenthi & Wildt, 2004). Regular, reliable access to individual conditioned animals provides the opportunity for application of daily transrectal ultrasonography, a technique that has enabled serial observation of ovarian follicle development and correlation of ovarian, endocrine and behavioral changes in many large domestic (Adams, 1999) and non-domestic animals (Adams *et al.*, 1991).

In cattle, ovarian follicles develop in waves during the estrous cycle (Knopf *et al.*, 1989, Pierson & Ginther, 1987). A wave of follicle development is characterized by the synchronous development of a group of small follicles that grow in diameter at an equal rate until one follicle is selected to continue growing while the rest regress. The selected follicle is functionally dominant over the subordinate follicles and is responsible for the cessation of growth and regression of the subordinate follicles (Adams *et al.*, 1993, Ginther *et al.*, 1989a,

Ko *et al.*, 1991, Pierson & Ginther, 1988). There are two or three waves of follicle development during the bovine estrous cycle (Ginther *et al.*, 1989b). Although follicular waves were not defined as such in previous studies in deer, a wave pattern was inferred by ultrasonographic detection of two to four large (ostensibly dominant) follicles during the estrous cycle in fallow deer (Asher, 1985), and one to three large follicles during the estrous cycle in red deer (Asher *et al.*, 1997).

Until recently, studies on ovarian function in wapiti have been restricted to post-mortem examination (Morrison, 1960b) or rectal palpation (Glover, 1985), and of the studies done in red deer, only one involved the use of ultrasonography (Asher *et al.*, 1997). In a recent critical study of ovarian function during the anestrous season (McCorkell *et al.*, 2004), transrectal ultrasonography was used to document that ovarian follicle development in wapiti occurs in a wave-like pattern characterized by the synchronous growth of a group of follicles from which a single follicle is selected and continues to grow while the others regress.

The objective of the present study was to characterize the temporal pattern of follicle and luteal development in relation to changes in circulating concentrations of progesterone, follicle stimulating hormone, and estradiol during the estrous season in wapiti.

2.3 Material and methods

Data were gathered from 13 wapiti hinds that were located on a farm near Saskatoon, Saskatchewan (52°07'N, 106°38'W). The hinds ranged in age from 26 months to 13 years (5.0 ± 2.7 years; mean ± SEM). The animals were kept in a one hectare pen and were fed alfalfa /brome grass hay. The hinds and the stag were maintained in separate but adjacent pens; i.e., two fences seven meters apart separated the pens.

Prior to examination, the hinds were moved from the pen to a handling facility via a 70 meter-long alley. Once in the facility, the hinds were restrained in a squeeze chute, without tranquilization, and the ovaries were examined by manual transrectal ultrasonography (Aloka SSD500, Instruments for Science and Medicine, Vancouver, Canada) using a 7.5 MHz linear-array transducer. Detailed drawings of the ovaries were made to record the number, diameter and relative position of follicles and the corpus luteum. The drawings were used to tabulate the number of follicles ≥ 4 mm within the pair of ovaries of each hind for each day of the examination period and to construct diameter profiles of uniquely identified follicles from their first appearance at 4 mm in diameter until they could no longer be uniquely identified (regressed to ≤ 4 mm; Knopf *et al.*, 1989, McCorkell *et al.*, 2004). The hinds were examined once daily until two successive ovulations had been recorded (Day 0 = first ovulation). An ovulation was defined as having occurred if a follicle ≥ 8mm in diameter identified during the previous day's examination was not present on the

subsequent day, and was confirmed by detection of a corpus luteum (CL) at the same location within the next 3 days.

2.3.1 Blood samples and hormone assays

Blood samples were collected daily from 10 hinds (three hinds were not amenable to daily blood collection) via jugular venipuncture using an 18-gauge 3.8 cm needle and a 10 ml vial without anticoagulant. Blood samples were kept chilled but prevented from freezing until centrifugation at 1500g for 10 minutes. The serum was removed and stored at -20°C.

Serum progesterone concentration was measured by a sequential competitive immunoassay using a polyclonal rabbit antiprogesterone-coated bead. Levels of progesterone were determined by measurement of chemiluminescence (Immulyte, Diagnostic Products Corporation, Los Angeles, California). To verify the assay for progesterone in wapiti serum, dilutions were made using carbon-stripped wapiti serum to which known amounts of progesterone were added in a serial fashion from 0.25 ng/ml to 64 ng/ml. The minimum detectable concentration was 0.2 ng/ml. The range of the standard curve was 0.25 ng/ml (80% ligand labeled progesterone) to 13.92 ng/ml (20% ligand labeled progesterone). To rule out the possibility that an interfering substance may have been removed from the carbon-stripped wapiti serum, another sample of wapiti serum, known to contain a high level of progesterone, was serially diluted with carbon stripped wapiti serum and yielded a line with a slope parallel to the dilution conducted using carbon-stripped serum alone. Intra-assay coefficients

of variation were 11.2% for the low reference sample and 7.4% for the high reference sample. The inter-assay coefficients of variation were 14.7% and 5.5% for the low and high reference samples, respectively (n = 10 assays).

Serum concentrations of FSH were determined using a previously published radioimmunoassay method (Rawlings *et al.*, 1984). The assay was validated for use in wapiti serum by the serial dilution of a wapiti serum sample from an estrous hind, which resulted in a parallel curve to the ovine standard. The minimum detectable limit of the assay was 0.25 ng/ml. The range of the standard curve was 0.65 ng/ml to 3.97 ng/ml. Intra-assay coefficients of variation were 3.1% for the low reference sample and 15.8% for the high reference sample. The inter-assay coefficients of variation were 14.8% and 6.3% for the low and high reference samples, respectively (n = 3 assays).

Serum concentrations of estradiol were determined on ether-extracted serum samples using a previously published radioimmunoassay method (Joseph *et al.*, 1992). The assay was validated for wapiti serum by obtaining a serial dilution curve parallel to the ovine standard from a wapiti serum sample from an estrous hind. The minimum detectable limit of the assay was 1 pg/ml and the range of the standard curve was 10.1 to 190.3 pg/ml. Intra-assay coefficients of variation were 18.9% for the low reference sample and 6.1% for the high reference sample. The inter-assay coefficients of variation were 12.5% and 13.7% for the low and high reference samples, respectively (n = 10).

2.3.2 Data analysis

A wave of follicle development was defined as the synchronous growth of a group of small follicles. The dominant follicle was defined as a follicle that attained a diameter ≥8 mm and exceeded the diameter of all others, and selection was defined as the day the dominant follicle became and remained larger than any of its cohorts. The day the dominant follicle of a wave was first detected at 4 mm in diameter was defined as the day of wave emergence.

For the purposes of analysis and illustration, data for day-to-day profiles were centralized to the day of wave emergence and divided into intervals encompassing successive wave emergence. Data for two-wave, three-wave and four-wave interovulatory intervals were considered separately. For the first and last waves respectively, data included several days before and after ovulation. For all other waves, data included an interval of several days before and after the mean day of wave emergence (indicated by interrupted x-axis lines in Fig. 2.1 to 2.3). Peaks in serum FSH and estradiol concentrations for individual animals were defined as a rise in FSH or estradiol over at least two consecutive days, followed by a decline for at least two consecutive days. Only data from hinds that contained definable peaks in serum FSH and estradiol concentration were considered in analysis involving peaks in serum FSH and estradiol (n = 13 waves for FSH and n = 11 waves for estradiol). Serial data were examined by analysis of variance for repeated measures using the PROC MIXED procedure of the Statistical Analysis System (SAS System 8, Cary, N.C.) to determine the effect of time (e.g., follicle number and diameter). Non-serial data were

examined for the effect of wave (i.e. Wave 1, 2, etc.) and wave pattern (i.e. two-, three-, and four-wave interovulatory intervals) by analysis of variance. The correlations between follicle diameter and follicle number, CL diameter and progesterone concentration, and follicle number and FSH concentration, respectively, were analyzed using the PROC CORR procedure of SAS. All data are presented as the mean ± the standard error of the mean (SEM).

2.4 Results

2.4.1 Follicular dynamics

There were non-random changes in follicle numbers (≥ 4 mm) and in the diameter of successive large follicles ($P < 0.05$) detected per day. An inverse relationship was found between follicle numbers and the diameter of the largest follicle (r –0.3, $P < 0.001$). Periodic changes in follicle numbers in temporal relation to emergence and growth of the largest follicle were taken as a wave pattern of follicle development. Hence, the largest follicle is hereafter referred to as the dominant follicle of a wave and all others as subordinates.

The overall mean interovulatory interval (IOI) was 21.3 ± 0.1 days, but the duration of the IOI differed ($P < 0.05$) according to the number of follicular waves that developed (Table 1). Of the 13 hinds, two waves of follicular development were detected during the IOI in six, three waves in five, and four waves in two. The IOI was shortest ($P < 0.05$) in the two-wave pattern. The characteristics of follicular development are compared among two-, three-, and four-wave patterns in Table 1. The intervals between waves in two-wave IOI

and the first interwave interval of three-wave IOI were similar in duration, but were significantly longer than the remaining interwave intervals of three-wave IOI and all intervals in four-wave IOI. The maximum diameter of the dominant follicle was similar between waves in two-wave IOI, but the maximum diameter of the first wave was greater ($P < 0.05$) than that of subsequent waves in three- and four-wave IOI. Relationships between the number of follicles ≥ 4 mm and the diameter profiles of successive dominant follicles in two-, three-, and four-wave IOI are illustrated in Fig. 2.1, 2.2, and 2.3.

Table 2.1. Comparison of follicular dynamics in wapiti with two, three, and four waves of follicle development during the estrous cycle (mean ± SEM; Day 0 = ovulation).

Number of waves	Interovulatory interval	Follicle wave	Day of wave emergence	Interwave interval	Maximum diameter of dominant follicle
2 (n = 6)	20.0±0.2[a]	1	0.0±0.4	10±0.1[a]	12.5±0.3[a]
		2	10.2±0.3	10±0.2[a]	11.7±0.2[a]
3 (n = 5)	22.2±0.3[b]	1	0.2±0.4	9.2±0.2[a]	10.4±0.1[a]
		2	9.2±0.7	6.2±0.2[b]	9.4±0.1[b]
		3	15.6±0.8	6.6±0.3[b]	9.2±0.1[b]
4 (n = 2)	23.0±0.7[b]	1	-1.0±0.0	7.0±0.0[b]	11.5±0.2[a]
		2	6.0±0.0	6.5±0.4[b]	9.5±0.4[b]
		3	12.5±0.5	4.0±0.7[b]	8.5±0.4[b]
		4	16.5±0.5	6.5±0.4[b]	9.5±0.4[b]

(a and b) Values with different superscripts within a column are different ($P < 0.05$)

The diameter profile of the dominant follicle of the first two waves in two- and three-wave patterns is compared in Fig. 2.4. The diameter profile of the first dominant follicle in two-wave patterns was not different to that of three-wave patterns until Day 10 ($P < 0.05$) when the diameter of the latter began to decrease. The profile of the dominant follicle of the second anovulatory wave in

three-wave patterns was smaller ($P < 0.05$) than that of the first wave in both two- and three-wave patterns.

Fluctuations in serum FSH concentration occurred in a periodic fashion in both two- and three-wave IOI ($P < 0.05$). Minima in FSH concentration were detected when follicle numbers were at or near their peak, and maxima in FSH were detected when follicle numbers were at or near their trough (Fig. 2.1 B, 2.2 B). Serum FSH concentration was negatively correlated with follicle numbers in two- and three-wave IOI ($r = -0.36$, $r = -0.21$, respectively; $P < 0.05$). Blood samples from hinds with four waves of follicle development were only available from one animal; therefore, hormone measurements are presented for completeness (Fig. 2.3 B) but were not included in statistical analyses.

In hinds with two follicular waves during the IOI, two peaks ($P < 0.05$) in serum FSH concentration were detected, the first on Day 0.0 ± 0.6 and the second on Day 10.6 ± 1.2. In hinds with three follicular waves, three peaks ($P < 0.05$) in serum FSH were detected on Day -0.3 ± 0.5, Day 8.3 ± 0.5, and Day 15.0 ± 0.4. The day of the second peak in FSH concentration tended to be later ($P = 0.09$) in hinds with two waves of follicle development compared to hinds with three waves. Serum FSH concentrations were determined in only one hind that had four waves of follicle development, and four peaks in FSH concentration were detected (Day -1, Day 7, Day 12, and Day 17). The maximum FSH concentration detected per wave was higher ($P < 0.05$) for the first follicular wave, associated with ovulation (0.61 ± 0.03 ng/ml), than for Waves 2 and 3 (0.45 ± 0.04 and 0.40 ± 0.4 ng/ml, respectively).

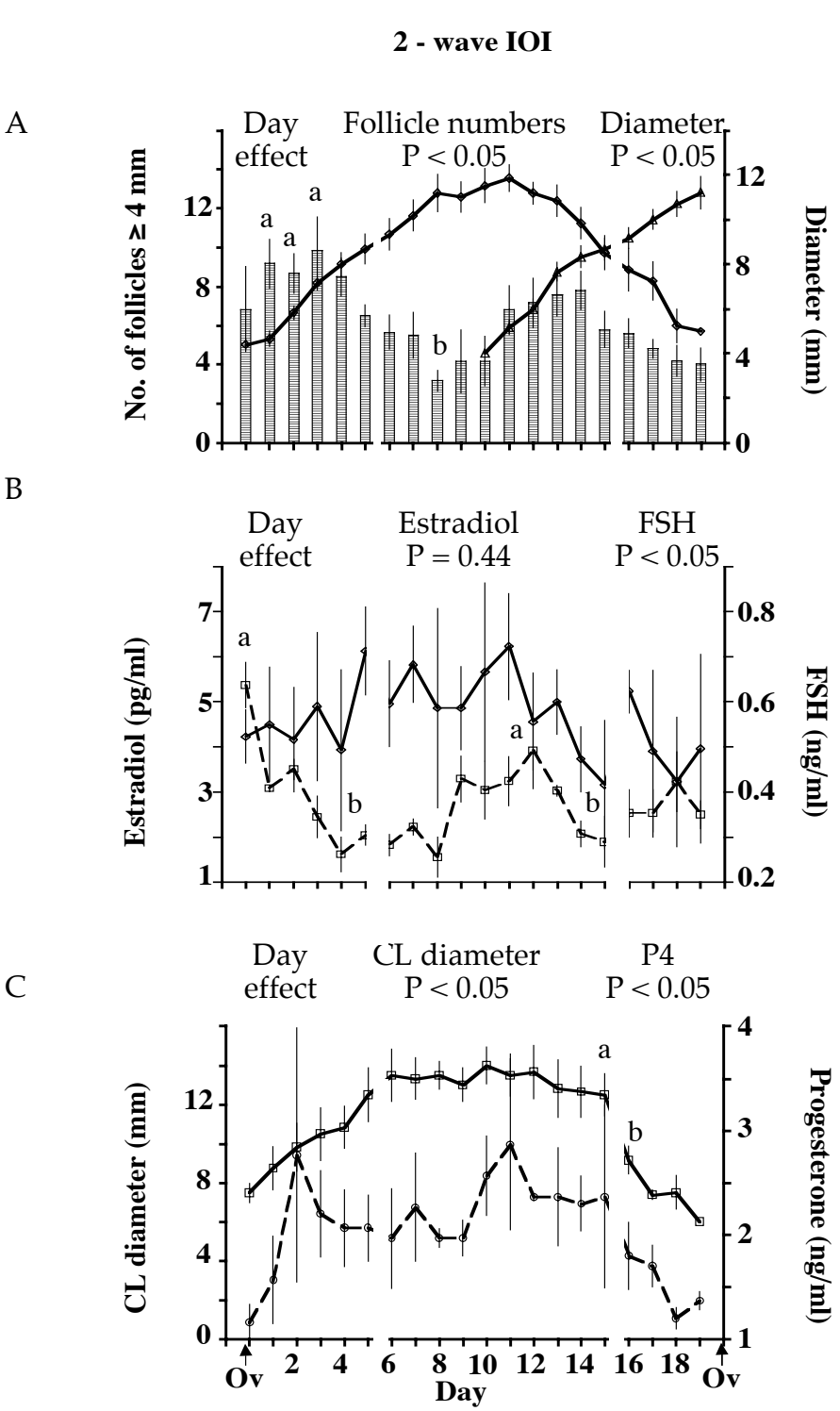

Figure 2.1 Follicle and luteal dynamics (mean ± SEM) in wapiti (n = 6) with two follicular waves during the interovulatory interval: (A) number of follicles ≥ 4 mm in both ovaries (bars) and diameter profiles of successive dominant follicles (—), (B) serum concentrations of estradiol (—; n = 4) and FSH (- -; n = 4), (C) CL diameter (—; n = 6) and serum progesterone concentrations (- -; n = 3).
(a and b) Among days, values with different superscripts are different (P < 0.05).

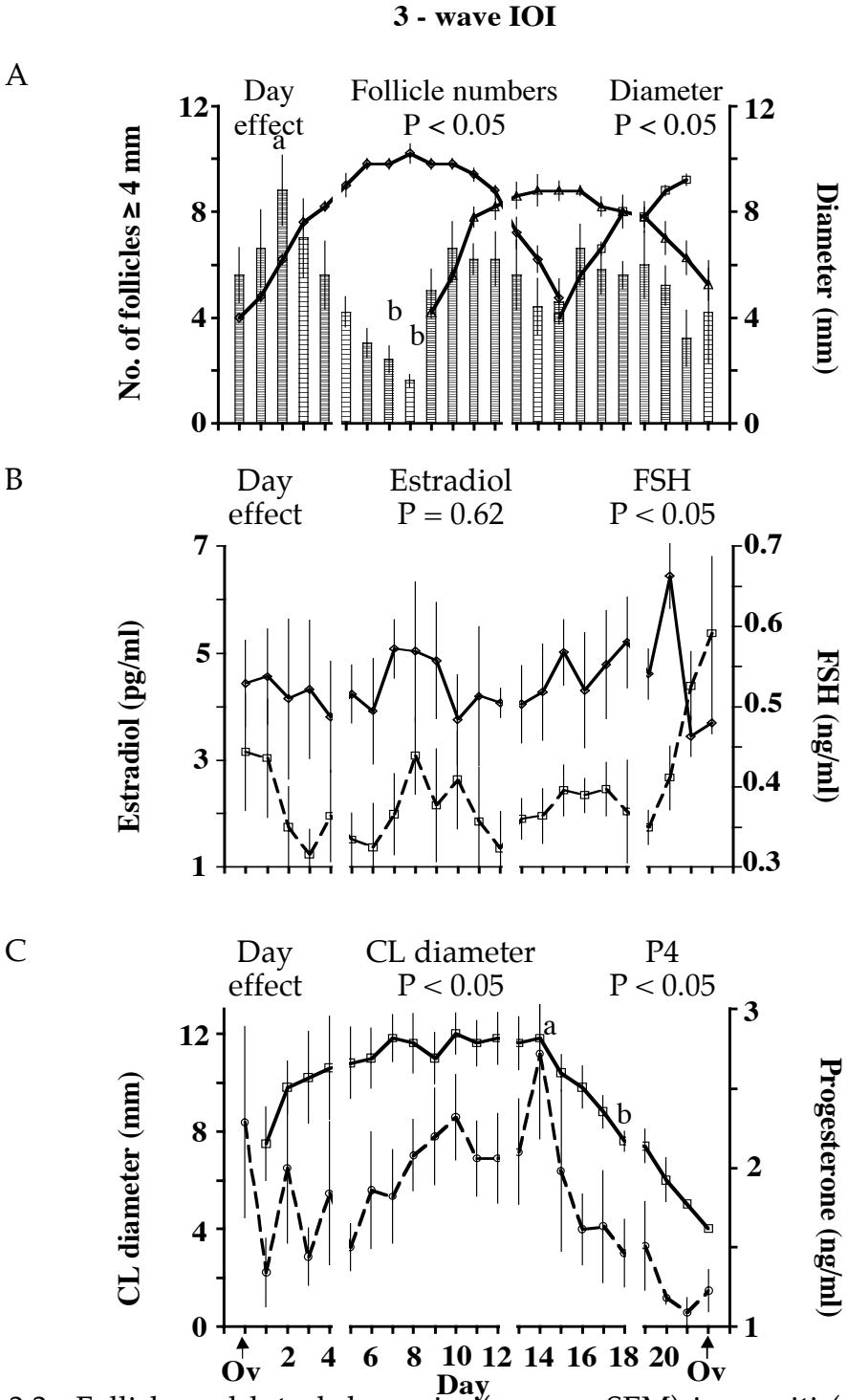

Figure 2.2. Follicle and luteal dynamics (mean ± SEM) in wapiti (n = 5) with three follicular waves during the interovulatory interval: (A) number of follicles ≥ 4 mm in both ovaries (bars) and diameter profiles of successive dominant follicles (—), (B) serum concentrations of estradiol (—) and FSH (- -), (C) CL diameter (—) and serum progesterone concentrations (- -).
(a and b) Among days, values with different superscripts are different (P < 0.05).

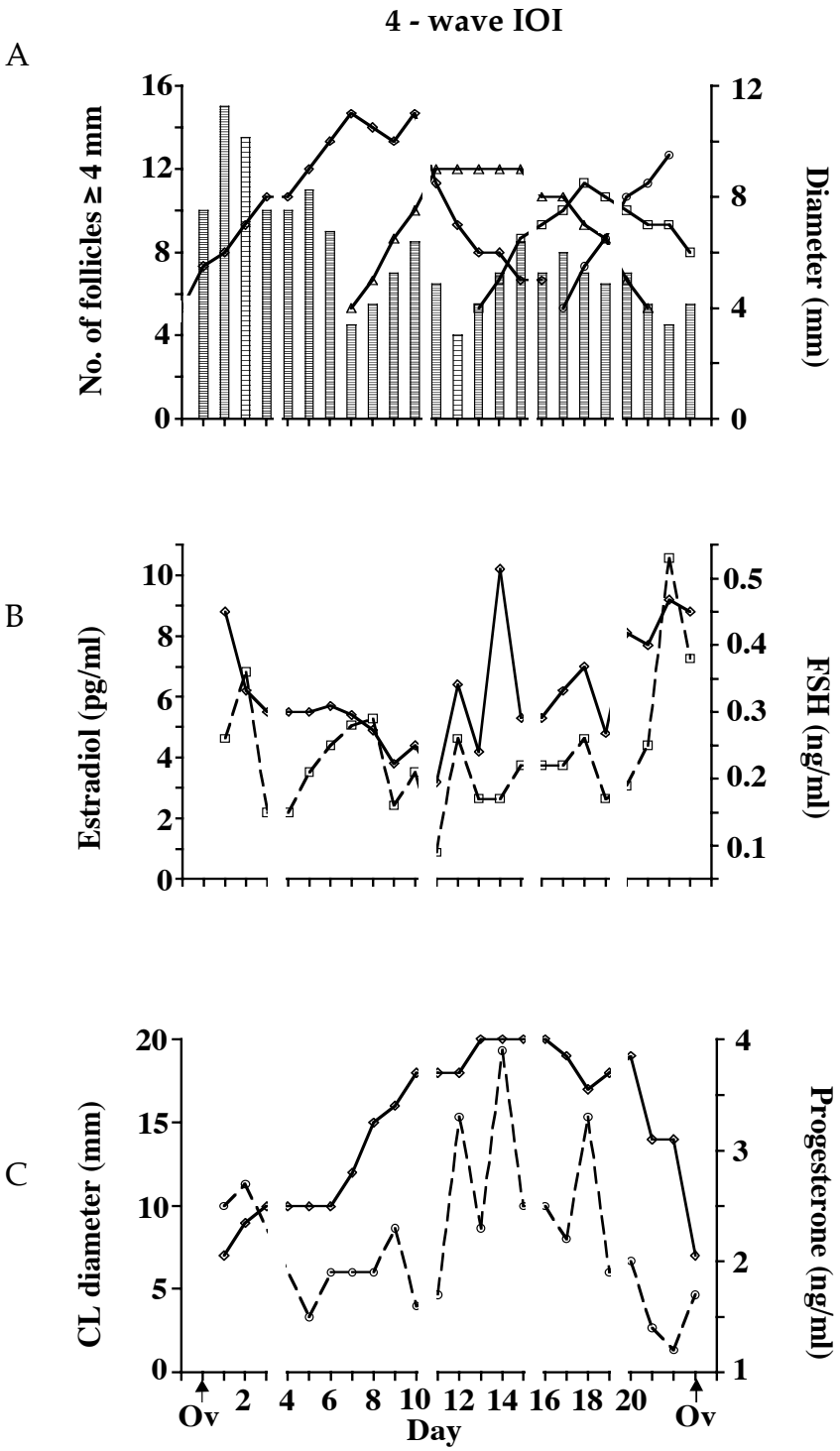

Figure 2.3. Follicle and luteal dynamics (mean ± SEM) in wapiti (n = 2) with four follicular waves during the interovulatory interval: (A) number of follicles ≥ 4 mm in both ovaries (bars) and diameter profiles of successive dominant follicles (—), (B) serum concentrations of estradiol (—; n = 1) and FSH (--; n = 1), (C) CL diameter (—; n = 2) and serum progesterone concentrations (- -; n = 1).

To examine the relationship between FSH concentration and ovulation, serum FSH concentrations were analyzed by centering the data to the first day of maximum FSH concentration detected near the time of ovulation (Fig. 2.5A). Ovulations followed a non-random pattern ($P < 0.05$); all ovulations occurred on the day of or the day after maximum serum FSH concentration. To examine the relationship between FSH concentration and follicle wave emergence, data for all waves were centered to the day of peak serum FSH concentration (Fig. 2.5B). The mean peak in FSH concentration was higher ($P < 0.05$) than the FSH concentration 2 days before and 1 day after the peak. The majority of follicular waves (77%) emerged ± 1 day of the peak in FSH concentration, but all emerged ± 2 days of the peak.

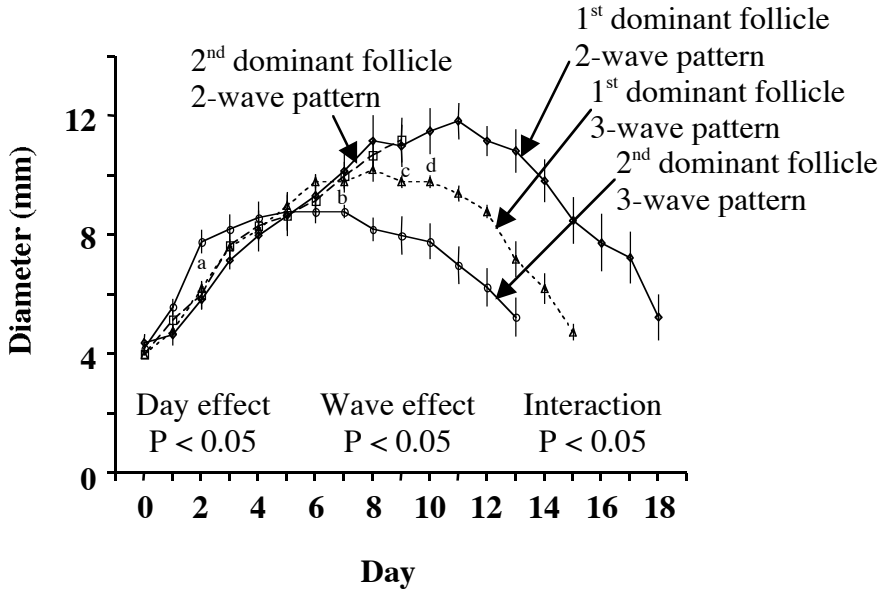

Figure 2.4. Comparison of dominant follicle diameter profiles (mean ± SEM) of the first two waves in wapiti with two- versus three-wave patterns during the estrous cycle.
[a] The second dominant follicle in three wave cycles is larger than the others ($P < 0.05$). [b] The second dominant follicle in three wave cycles is smaller than the others ($P < 0.05$). [c] The first dominant follicle in three wave cycles is smaller than the second dominant follicle in two-wave cycles ($P < 0.05$). [d] The first dominant follicle in three wave cycles is smaller than the first dominant follicle in two-wave cycles ($P < 0.05$).

Day-to-day changes in serum estradiol concentrations were not significant for either two- or three-wave IOI (Fig. 2.1, 2.2, 2.3). However, when data were centered on the first day of maximum estradiol concentration near the time of ovulation (Fig. 2.6A), there was a non-random pattern in the distribution of ovulations ($P < 0.05$). Of the 10 ovulations detected, 80% occurred 1 or 2 days after the peak in serum estradiol concentration. To examine the relationship between serum estradiol concentration and follicle wave emergence, the data were centered on the peak concentration of estradiol for all estradiol peaks and all hinds (Fig. 2.6B). Serum estradiol concentration on the day of the peak was higher ($P < 0.05$) than on proceeding and following days; however, the emergence of follicular waves was not restricted to any particular day relative to the peak.

The dominant follicle emerged, on average, 0.4 ± 0.1 days before the largest subordinate follicle and 0.8 ± 0.1 days before the second largest subordinate follicle. The largest follicle on Day 0 was the one destined to become dominant in 12 of 35 (34%) waves, on Day 1 in 18 of 35 (51%) waves, on Day 2 in 27 of 35 (77%) waves, and on Day 3 in 33 of 35 (94%) waves. On average, the follicle destined to become dominant was larger ($P < 0.05$) than the largest subordinate follicle 1 day after emergence at 4 mm in diameter. This coincided with the first significant decrease in serum FSH concentration (Fig. 2.5).

The interval from emergence of the ovulatory wave to ovulation was shorter ($P < 0.05$) in three- and four-wave hinds than in two-wave hinds (6.6 ± 0.5 days versus 10.5 ± 0.5 days), and the maximum diameter of the ovulatory follicle was

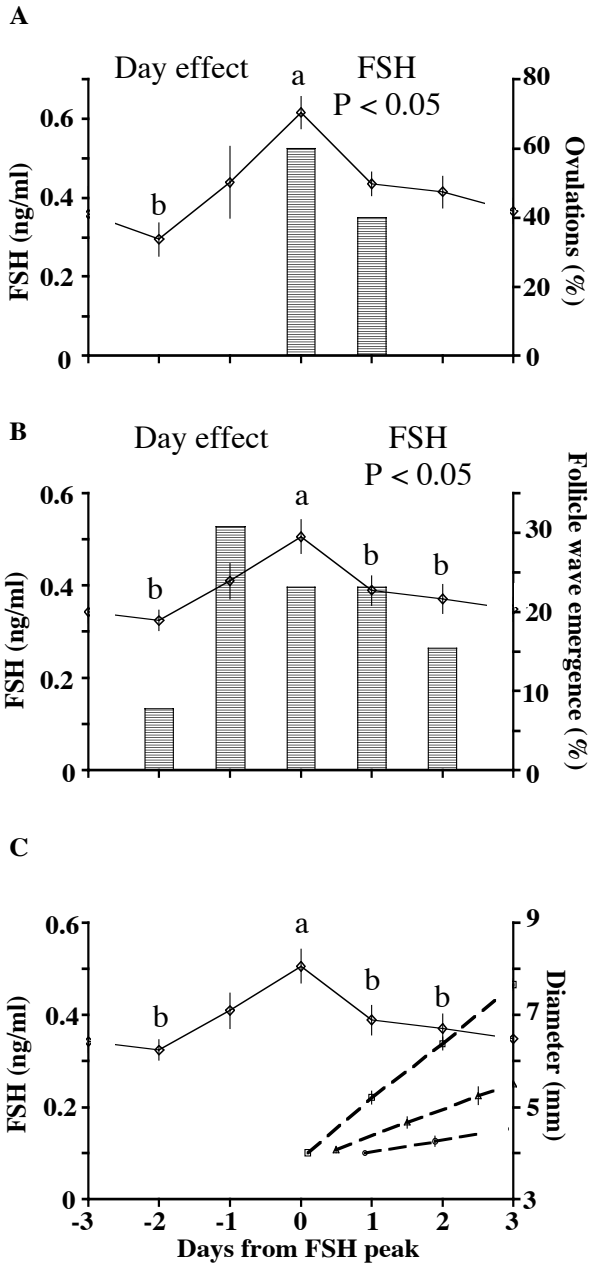

Figure 2.5. Serum FSH concentrations in wapiti (—; mean±SEM; n = 10) obtained when: (A) the data were centralized to the peak of FSH (Day 0) detected near the time of ovulation (bars indicate the percentage of ovulations detected each day), (B) the data were centralized to the peak of FSH (Day 0) detected near the time of follicular wave emergence (all waves combined, n = 13; bars indicate the percentage of waves that emerged on each day), and (C) the data were centralized to the peak of FSH (Day 0) detected near the time of follicular wave emergence (all waves combined, n = 13) and plotted in relation to the diameter profiles of the three largest follicles in the wave. (a and b) Among days, serum FSH values with different superscripts are different ($P < 0.05$).

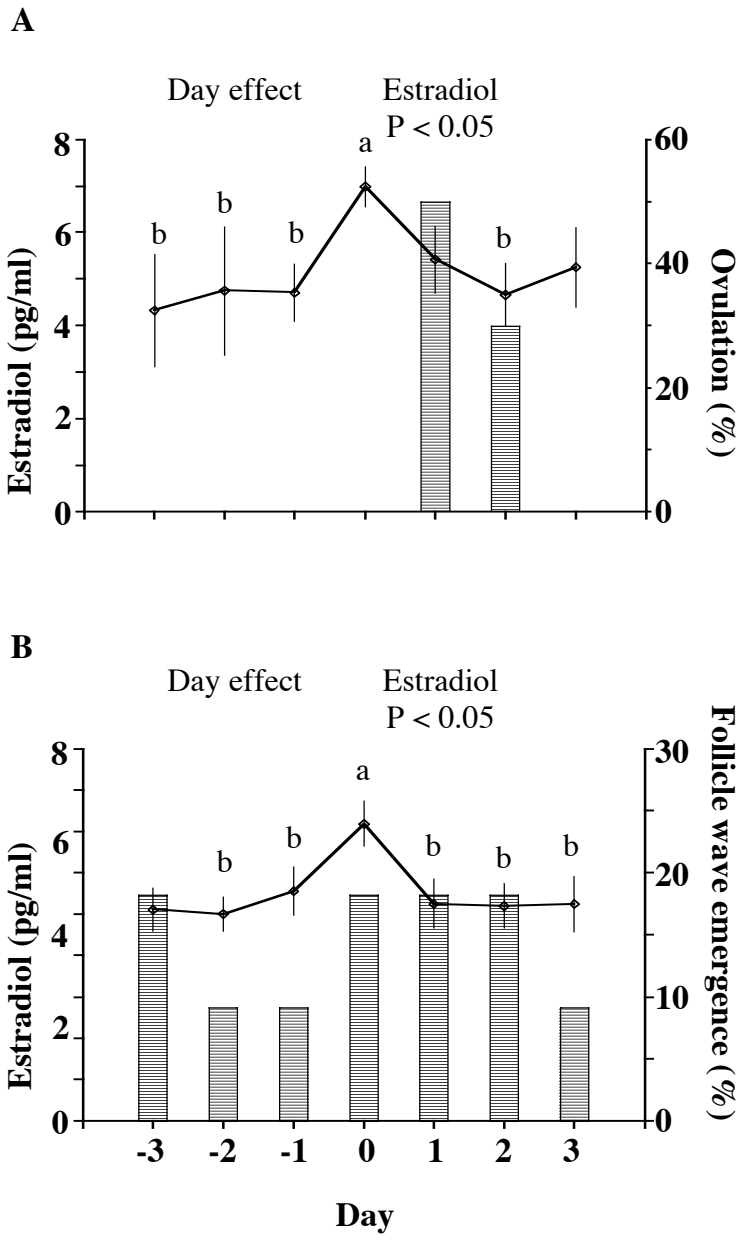

Figure 2.6. The profile of daily serum estradiol concentrations (—; mean±SEM; n = 10) obtained when: (A) the data were centralized to the peak of estradiol (Day 0) that occurred near the time of ovulation (percentage of ovulations occurring on each day indicated by bars), (B) the data were centralized to the peak of estradiol (Day 0) that occurred near the time of wave emergence (percentage of follicular waves emerging on each day; n = 11 waves).
(a and b) Among days, serum estradiol values with different superscripts are different (P < 0.05).

smaller ($P < 0.05$) in three- and four-wave hinds than in two-wave hinds (9.0 ± 0.3 mm versus 11.5 ± 0.3 mm).

2.4.2 Luteal dynamics

Only singleton ovulations were detected and only one CL per hind was identified during the IOI. The growth and demise of the CL was detected by changes ($P < 0.05$) in ultrasonically detected diameter during the IOI (Figs 2.1, 2.2, 2.3). The CL was detected on Day 1 in seven hinds, and by Day 2 in the remaining six hinds (Table 2). Fluid-filled central cavities were present in 11 of 13 CL and ranged from 2 mm to 17 mm in diameter (5.1 ± 1.1 mm in diameter on Day 10). The diameter of the fluid-filled cavities remained static from Day 6 (5.0 ± 1.0 mm) to Day 12 (5.2 ± 1.4 mm) and then declined at a mean rate of 0.7 mm/day. In all but four hinds, the cavities became undetectable before the onset of luteolysis. The regressing CL was identified ultrasonographically until one ($n = 4$) or two ($n = 6$) days before ovulation or the day of ovulation ($n = 3$). The regressing CL was not detected after the day of ovulation in any of the hinds.

Serum progesterone concentrations (Figs. 2.1, 2.2, 2.3) changed during the interovulatory interval but were not different between two- and three-wave patterns (day effect, $P < 0.05$; wave pattern effect, $P = 0.6$; interaction, $P = 0.6$). Serum progesterone concentrations were lowest during the period encompassing 2 days before and 1 day after ovulation (1.2 ± 0.1 ng/ml) and

began to increase ($P < 0.05$) on Day 3.5 ± 0.9. Progesterone concentrations were maximal at 3.4 ± 0.3 ng/ml on Day 12.9 ± 0.8 and then decreased ($P < 0.05$) sharply on Day 16.4 ± 0.6. Serum progesterone concentrations were positively correlated with CL diameter ($r = 0.3$, $P < 0.05$). The decrease in progesterone concentration during luteolysis preceded a precipitous drop in CL diameter by 1 day; progesterone concentrations began to decline 7.0 ± 0.6 day before ovulation and the CL began to regress 5.7 ± 0.6 day before ovulation.

Table 2. Comparison of luteal dynamics in wapiti with two, three, and four waves of follicle development during the estrous cycle (mean ± SEM; Day 0 = ovulation).

	Number of follicle waves during estrous cycle		
	2	3	4**
Number of wapiti	6	5	2
Maximum CL diameter (mm)	15.5±0.5	13.4±0.7	16.5±2.5
Maximum progesterone concentration (ng/ml)*	3.7±0.4	3.2±0.4	3.9
Day of initial CL detection	1.0±0.4	1.6±0.2	1.5±0.5
Day of maximum CL diameter	15.5±1.1	13.4±1.5	16.5±3.5
Day of onset of CL regression	15.7±0.7	16.4±0.7	18.5±2.5
Day of first rise in serum progesterone concentration*	2.3±0.3	4.8±1.4	1
Day of maximum serum progesterone concentration*	11.3±1.8	13.6±0.8	14
Day of onset of decline in serum progesterone concentration*	14.7±0.9	15.4±0.7	17
Day when serum progesterone reached nadir	17.3±0.7	19.6±0.8	22

No significant differences for any end point.
*Data from n = 3, n = 5, n = 1 in the respective groups.
** Data from four-wave interovulatory intervals not included in statistical analyses

2.5 Discussion

The pattern of follicle development observed during the estrous cycle of wapiti was characterized by the regular and synchronous development of a group of follicles in temporal succession to a surge in serum FSH concentration. Ovarian follicular waves became apparent as such when follicles emerged at a diameter of 4 mm on the day FSH concentrations reached a peak. From each wave of ovarian follicles, a single follicle was selected to continue to grow and become the largest follicle within the pair of ovaries while the other follicles of the cohort regressed. The regular development of follicular waves began immediately following ovulation. The number of waves of follicle development observed during one IOI varied from two to four. However, two or three waves of follicle development were the most common and occurred with similar frequency. The observational period encompassed only one IOI; hence, it was not possible to determine if an animal that has two waves of follicle development during an IOI will consistently have two waves of follicle development for every IOI.

The wave pattern of follicle development described in this report is similar to that proposed by Thomas and Cowan in 1975 after their histological study of ovaries recovered from hunter-killed black-tailed deer (*Odocoileus hemionus columbianus*) (Thomas & Cowan, 1975). They proposed that the interval between successive large follicles was 8 to 9 days with estrus occurring every second or third follicular cycle, depending on the functional life of the associated CL. The findings are remarkably consistent with findings of the present study given that

they were working with static tissues and non-serial data. The wave pattern is also similar to that reported in wapiti during the anestrous season (McCorkell *et al.*, 2004) and the pattern of follicle development reported in cattle (Ginther *et al.*, 1989b, Knopf *et al.*, 1989), sheep (Ginther *et al.*, 1995), goats (Ginther & Kot, 1994) and llamas (Adams *et al.*, 1990).

The pattern change in serum FSH concentration supported the concept of a feed-back loop between the ovary and the pituitary, as reported in cattle (Adams *et al.*, 1992b), where FSH is suppressed by products from growing follicles and FSH surges when these products decrease as the follicles stop growing and become atretic (Adams *et al.*, 1992b). In wapiti, the increase in serum FSH concentration began 2 days before wave emergence and peaked on the day of or the day before wave emergence similar to that reported in cattle and sheep (Adams *et al.*, 1992b, Ginther *et al.*, 1995). The periovulatory FSH peak, preceding emergence of Wave 1, occurred on the day of or the day before ovulation and was higher than that preceding any other wave.

The trough in FSH concentration occurred on the day of or 1 day before the newly selected dominant follicle became the largest follicle within the ovaries. Thereafter, circulating FSH concentrations began to rise, suggesting that the ability to suppress FSH was lost at about the time the next dominant follicle gained a diameter advantage over all other follicles within the pair of ovaries.

In most hinds (9 of 13), the first interwave interval was longer than that of all subsequent waves and longer than that reported in anestrous hinds (~ 7 d)

(McCorkell *et al.*, 2004). In hinds with two follicular waves, the interwave interval was the same for the first and second waves (~10 days) and this interval was not different from that of the first wave in three-wave IOI (P = 0.16). All other interwave intervals were similar in length to those observed during the anestrous season (McCorkell *et al.*, 2004).

The dominant follicle of the first wave reached the largest diameter of any dominant follicle in subsequent waves. It emerged when progesterone concentrations were at their lowest and developed for 1 to 2 days before progesterone concentrations began to increase. In cattle, progesterone inhibited the growth of the dominant follicle in a dose-dependent manner (Adams *et al.*, 1992a). At the time of ovulation, and for several days after, progesterone concentrations were low. Therefore, this was a privileged time for follicle development; subsequent waves emerged in an environment with relatively higher progesterone concentrations. In a high-progesterone environment, follicle growth is reduced in cattle (Adams *et al.*, 1992a, Ginther *et al.*, 1989b), consistent with the observation in hinds where the maximum diameter of the dominant follicle of Waves 2, 3, and 4 was smaller than that of Wave 1. The smaller diameter of the ovulatory follicle in three- and four-wave hinds was associated with a shorter interval from emergence to luteal regression and ovulation. As reported in cattle (Ginther *et al.*, 1989b), emergence of the ovulatory follicle occurred before luteolysis.

The length of the IOI was significantly longer in three-wave cycles than in two-wave cycles. In cattle, the difference between two and three waves of follicular

development has been attributed to the increased length of the luteal phase in three-wave animals (Driancourt, 2001, Ginther *et al.*, 1989b). In wapiti, however, no differences in CL diameter profiles or serum progesterone concentrations were detected between two- and three-wave cycles. Rather than a delay in luteal regression, the manifestation of a three-wave versus two-wave cycle in wapiti may be related to the persistence of the first dominant follicle relative to the growth of the second dominant follicle. If the second dominant follicle became the largest within the pair of ovaries before the onset of CL regression, it did not go on to ovulate. Instead it began to regress and a new follicular wave emerged (i.e., three-wave cycle). Conversely, if the second dominant follicle was inferior in diameter to the dominant follicle of the first wave when the CL began to regress, it would continue to grow and ultimately ovulate (i.e., two-wave cycle). The second dominant follicle exceeded the diameter of the first dominant follicle on Day 15.7 ± 0.4 and Day 12.9 ± 0.8 in two- and three-wave cycles, respectively ($P < 0.05$). A similar pattern has been described in cattle with two and three waves of follicle development (Ginther *et al.*, 1989b).

The growth and development of the second dominant follicle is related to the suppression of FSH by the first follicular wave. In this study, the FSH surge preceding the second wave of follicle development tended to occur ($P = 0.09$) sooner in three-wave than two-wave cycles. This suggests that the properties of the follicles of the first wave may be responsible for determining the two- or three-wave pattern.

The maximum diameter of the dominant follicle in this study ranged from 9 to 14 mm consistent with a previous report of 8 to 11 mm in hinds killed on known days after mating with a vasectomized stag (Morrison, 1960b). Except for the period of 1 to 3 days after ovulation, at least one large follicle (> 7 mm) was present within the pair of ovaries throughout the IOI. This is consistent with previous ultrasound studies in wapiti (McCorkell *et al.*, 2004), red deer (Asher *et al.*, 1997) and fallow deer (Asher *et al.*, 1999), but in contrast to findings of another study in red deer in which the presence of a large follicle was reported throughout the estrous cycle (McLeod *et al.*, 2001).

The pattern of follicle growth in wapiti in the present study was regular and synchronous, unlike the highly variable and non-synchronous pattern described in red deer (Asher *et al.*, 1997). Although not strictly defined, apparent wave emergence in red deer was reported to occur on Days 1 and 14 (Asher *et al.*, 1997), whereas, wave emergence in wapiti in the present study occurred on Days 0 and 10 in two-wave cycles and on Days 0, 9, and 16 in three-wave cycles. The interval from emergence to ovulation of the ovulatory follicle in two- and three-wave cycles in the present study (10.0 ± 0.5 and 6.6 ± 0.7 days, respectively) was similar to that reported in cattle (10.9 ± 0.4 and 6.8 ± 0.6 days, respectively; Ginther *et al.*, 1989b) and suggests that the process of follicular and oocyte maturation may be similar between these species. However, the same cannot be said when comparing follicle growth patterns of wapiti with sheep and goats. Unlike wapiti, the phenomenon of follicular dominance is not clear in sheep and goats and follicular dynamics are manifest in the occurrence of both major and minor waves (Adams, 1999). Furthermore, most estrous cycles in

sheep and goats are composed of four follicular waves (Ginther & Kot, 1994, Ginther *et al.*, 1995).

The CL in wapiti were ultrasonographically detectable as early as Day 1 in some animals and consistently detectable by Day 2 in all animals, unlike red deer in which detection was inconsistent (Asher *et al.*, 1997) and fallow deer where the CL could not be identified until Day 4 to 6 (Asher *et al.*, 1999). Fluid filled cavities within the CL were a common feature in wapiti in the present study, similar to those described in sheep (Schrick *et al.*, 1993), cattle (Sprecher *et al.*, 1989), fallow deer (Asher *et al.*, 1999) and previously in wapiti (Morrison, 1960b). Consistent with observations in fallow deer in which the fluid-filled cavities were gone by Day 16 to 18 (Asher *et al.*, 1999), the cavities in wapiti CL were undetectable by the onset of luteolysis. After ovulation, the regressing CL could no longer be identified in the present study, in contrast to a report in fallow deer in which luteal remnants were identified until Day 5 of the following IOI (Asher *et al.*, 1999).

As reported in a previous study (McCorkell *et al.*, 2004), the technique of transrectal ultrasonography enabled consistent visualization of both ovaries and allowed data collection on follicular and luteal dynamics in 100% of the examinations of all animals in the study. Results were obtained without surgical modification, as required in red deer (Asher *et al.*, 1997), and were more consistent than for examinations conducted in fallow deer (Asher *et al.*, 1999). The experimental approach used in the present study enabled detailed characterization of ovarian function during the estrous cycle in wapiti, and

provide justification for the proposition that wapiti provide a suitable model for research on ovarian dynamics of seasonally breeding, monovulatory cervids.

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3.0 OVARIAN FOLLICULAR AND LUTEAL DYNAMICS IN WAPITI DURING SEASONAL TRANSITIONS

3.1 Abstract

The transitions from the anovulatory to the ovulatory season (n = 20) and ovulatory to anovulatory season (n = 11) were monitored in wapiti by daily transrectal ultrasonography. The ovulatory season was 142 days, defined by the interval between the median date of the first (September 26) and last ovulations (February 15). In 17 of 20 observations (85%) the first interovulatory interval (IOI) was short (9.1 ± 0.3 days) compared with the IOI later in the breeding season (21.3 ± 0.1) and the last IOI of the season (21.2 ± 0.6 days). With one exception, the short IOI were composed of only 1 wave of follicular development; the remaining IOI during the ovulatory season were composed of 2 or 3 waves. Maximum diameters of the first 2 ovulatory follicles were similar (11.3 ± 0.4 vs 11.3 ± 0.2 mm), but both were larger ($P < 0.05$) than the last 2 ovulatory follicles of the ovulatory season (10.3 ± 0.3 vs 10.1 ± 0.4 mm). Multiple ovulations were observed in 3 hinds on their first ovulation of the breeding season and in one hind at the second ovulation of the breeding season. Multiple ovulations were not observed at any other time during the study. Day-to-day profiles of CL diameter and plasma progesterone concentration were smaller ($P < 0.05$) for short IOI than for long IOI. Maximum diameter (12.8 ± 0.6 vs 12.5 ± 0.6 mm) and the diameter profile of the last CL of the season were not different

from that of the previous CL; however, the last CL was detected for a longer period (22.3 ± 1.2 vs 19.3 ± 0.7 days; $P < 0.05$). In summary, transition to regular estrous cycles was preceded by one short IOI (9 days). The characteristics of the last IOI of the breeding season were similar to those reported during the rut; i.e., there was no indication of cycle irregularities with the approach of the non-breeding season. Transition to anovulation was marked by a failure of the dominant follicle to ovulate after a typical luteal phase.

3.2 Introduction

Wapiti (*Cervus elaphus*), a subspecies of red deer, are one of 15 species of boreal or temperate deer (Lincoln, 1992). They have a seasonal reproductive pattern that ensures their calves are born at a time of year when food is available and the weather is favorable for their survival (Lincoln, 1983, Loudon & Brinklow, 1990). The seasonal pattern of reproduction is apparently under photoperiodic control (Lincoln, 1983, Loudon & Brinklow, 1990). Under the influence of lengthening days during late winter and spring, luteinizing hormone (LH) secretion is inhibited and wapiti enter a period of anestrus. The inhibition of LH secretion is lost under the influence of shortened day-length during late summer and fall, and the breeding season begins (Anderson & Barrell, 1998, Meikle & Fisher, 1996). The transitions into and out of the breeding season are marked by the resumption of ovulation in autumn and the cessation of ovulation in winter, respectively (Morrison, 1960). Wapiti are seasonally polyestrus (Glover, 1985, Lincoln, 1992, Morrison, 1960) and the onset of ovulatory cyclicity is distinguished by aggressive breeding behavior of stags in rut (Guinness *et al.*, 1971, Struhsaker, 1967). The end of the breeding season is not marked by distinct behavioral changes, and the characteristics of the transition to anestrus have not been as well studied.

In wapiti, ovarian follicles develop in waves during the breeding season (McCorkell *et al.*, 2006). A wave of follicle development is characterized by the synchronous development of a group of small follicles that grow at an equal rate until one follicle is selected to continue growing while the rest regress. The

selected follicle is functionally dominant over the subordinate follicles and is responsible for the cessation of growth and regression of the subordinate follicles (Adams *et al.*, 1993, Ginther *et al.*, 1989, Ko *et al.*, 1991, Pierson & Ginther, 1988). The majority of estrous cycles in wapiti (85%) are composed of 2 or 3 waves of follicle development and the remainder are composed of 4 waves (McCorkell *et al.*, 2006). Ovarian follicle development in wapiti also occurs in waves during the non-breeding season (McCorkell *et al.*, 2004); anovulatory waves emerge every 6 or 7 days, when the dominant follicle of the previous wave begins to regress.

The transition from the anovulatory to the ovulatory season was characterized by a short first estrous cycle (half the normal length) in some studies in red deer (*Cervus elaphus*; Asher *et al.*, 2000, Jopson *et al.*, 1990), fallow deer (*Dama dama*; Asher, 1985), white-tailed deer (*Odocoileus virginianus*; Harder & Moorhead, 1980) and Columbian black-tailed deer (*Odocoileus hemionus columbianus*; Thomas & Cowan, 1975). Others, however, did not detect a short estrous cycle during transition (Adam *et al.*, 1985, Guinness *et al.*, 1971). In a study involving serial transrectal palpation of wapiti (Glover, 1985), the author concludes that seasonal transition from anestrus to estrus was marked by irregular follicle development leading to estrous cycles of varying lengths that may or may not include ovulation. The author also reports that irregular circulating concentrations of progesterone that were only slightly elevated above detectable levels characterized the transition.

At the end of the ovulatory season in mid to late winter, the transition into the anovulatory season occurred over a longer time period (2 months) than the transition into the estrus season (Glover, 1985, Guinness *et al.*, 1971). In fallow deer and wapiti, the estrous cycle was reported to increase in length and become more irregular as the anovulatory season approached (Asher, 1985, Glover, 1985).

The objective of this study was to characterize the temporal pattern of ovarian follicular and luteal development in relation to changes in circulating concentrations of progesterone, follicle stimulating hormone (FSH), and estradiol during the periods of transition into and out of the ovulatory season in wapiti.

3.3 Materials and methods

The transition from anestrus to estrus was studied in 16 wapiti hinds, aged 2 to 14 years, over two successive seasons (n = 20 observations; 11 in year 1 and 9 in year 2). The transition from estrus to anestrus was studied in 11 hinds over one season. The hinds were maintained on a farm near Saskatoon, Saskatchewan (52°07′N, 106°38′W) with only natural light. They were fed alfalfa/brome hay in a 1-hectare pen that was separated from the stag's pen by 2 fences 7 meters apart. The hinds were moved daily from the pen through a 70 meter-long alley to the handling facility and restrained in a squeeze chute for examination (McCorkell *et al.*, 2001). The ovaries were examined by transrectal ultrasonography (Aloka SSD500, Instruments for Science and Medicine,

Vancouver, Canada) using a 7.5 MHz linear-array transducer. During each examination, a sketch of each ovary was made showing the size and location of follicles ≥ 4 mm in diameter and the corpus luteum. The drawings were used to tabulate the number of follicles ≥ 4 mm within the pair of ovaries of each hind for each day of the examination period and to construct diameter profiles of individually identified follicles from their first appearance at 4 mm until they could no longer be individually identified (regressed to ≤ 4 mm) (Knopf *et al.*, 1989, McCorkell *et al.*, 2004). An ovulation was defined as having occurred if a follicle ≥8 mm in diameter identified during the previous day's examination was not detected on the subsequent day, and a corpus luteum (CL) was identified in its place within the next 3 days (McCorkell *et al.*, 2006).

The ovaries were examined daily in September and October for evidence of the onset of the ovulatory season. To study the transition to anestrus from estrus, hinds were examined daily for the month of December and then every 3 or 4 days (Monday and Thursday), to detect imminent ovulation. When a regressing CL and a large growing follicle were detected, daily examinations were resumed until ovulation occurred, whereupon examinations were again done at 3- to 4-day intervals. On February 15, daily examinations began for all hinds in which a CL was detected. Daily examinations were maintained for at least 30 days after the last ovulation or until 10 days after the CL from the last ovulation regressed to a point where it was no longer detectable. The last examinations were completed on April 25.

3.3.1 Blood samples and hormone assays

A blood sample was collected at each examination from 15 animals (1 hind was not amenable to daily blood collection) during the fall transition, and from 7 animals during the winter transition. Samples were collected from the jugular vein using an 18 gauge, 3.8 cm needle into 10 ml vials without anticoagulant. Blood samples were kept chilled but prevented from freezing until centrifugation at 1500g for 10 minutes within 4 hours. The serum was removed and stored at -20°C.

Serum progesterone concentration was measured using a chemiluminescence assay (Immulyte, Diagnostic Products Corporation, Los Angeles, California) previously validated in wapiti (McCorkell *et al.*, 2006). Intra-assay coefficients of variation were 10% for the low reference sample and 7% for the high reference sample. Over 10 assays, the interassay coefficients of variation were 15% and 5% for the low and high reference samples, respectively.

Serum concentrations of FSH were determined using a previously published and validated radioimmunoassay (McCorkell *et al.*, 2006, Rawlings *et al.*, 1984). Intra-assay coefficients of variation ranged from 8.7% to 10.5%. The interassay coefficient of variation was 9.5% (n = 3).

Serum concentrations of LH were determined on selected samples using a previously published radioimmunoassay (Rawlings *et al.*, 1984). One sample taken the day after the first or last ovulation of the season, and another taken at

the middle of the luteal phase (Day 5 after the first ovulation and Day 11 after the last ovulation) were assayed. The assay was validated for use in wapiti serum by the serial dilution of wapiti serum from an estrous hind, which resulted in a parallel curve to an ovine standard. The range of the standard curve was 0.16 ng/ml to 0.95 ng/ml. Intra-assay coefficients of variation were 15% for the low reference sample and 2% for the high reference sample. Over two assays, the interassay coefficients of variation were 16% and 2% for the low and high reference sample, respectively.

Serum concentrations of estradiol were determined on ether-extracted serum samples using a previously published and validated radioimmunoassay (Joseph *et al.*, 1992, McCorkell *et al.*, 2006). Intra-assay coefficients of variation were 27% for the low reference sample and 12% for the high reference sample. The interassay coefficients of variation were 13% and 9% for the low and high reference samples, respectively (n = 10 assays).

3.3.2 Data analysis

A wave of follicle development was defined as the synchronous growth of a group of small follicles. The dominant follicle was defined as a follicle that attained a diameter ≥8 mm and exceeded the diameter of all others, and selection was defined as the day the dominant follicle became and remained larger than any of its cohorts. The day the dominant follicle of a wave was first detected at 4 mm in diameter was defined as the day of wave emergence.

For the purposes of analysis and illustration, data for day-to-day profiles were centralized to the day of wave emergence and divided into intervals encompassing wave emergence. Data for 2-wave and 3-wave IOI were considered separately. For the first and last follicle waves respectively, data included several days after and before ovulation. For all other waves, data included an interval of several days before and after mean day of wave emergence (indicated by broken lines in Figures 3.1 and 3.2). Peaks in serum FSH and estradiol concentrations for individual animals were defined as occurring when there was a rise in FSH or estradiol over at least two consecutive days, followed by a declined for at least 2 consecutive days. Serial data were examined by analysis of variance for repeated measures using the PROC MIXED procedure of the SAS System (SAS System 8, Cary, N.C.) to determine the effect of time (e.g. follicle number and day). Correlations were analyzed using the PROC CORR procedure of the SAS System. Non-serial data were examined for the effect of wave (i.e. wave 1, 2, etc.) and wave pattern (i.e. 2 and 3-wave IOI) by analysis of variance. All data are presented as the mean ± the standard error of the mean (SEM).

3.4 Results

3.4.1 Fall transition

The first ovulation was recorded on September 15 and all hinds had ovulated for the first time by October 7. Multiple ovulations were detected in 4 hinds (4 of 20 observations; 20%); 3 double ovulations were the first of the ovulatory season, the remaining was a triple ovulation and was the second of the season.

The maximum diameter of the first ovulatory follicle was 11.1 ± 0.3 mm in hinds with a single ovulation, which was larger ($P < 0.05$) than the maximum diameter of the first ovulatory follicle in hinds with double ovulations (9.9 ± 0.3 mm). The first interovulatory interval (IOI) in 17 of 20 cases was short and lasted 9.3 ± 0.4 days; 16 of the 17 short IOIs had only 1 wave of follicle development. The number of days the first ovulatory follicle was detected from its emergence at 4mm until it ovulated was 8.5 ± 1.1 days and was very similar to that of the second ovulatory follicle (9.1 ± 0.3 days) in hinds with a short IOI. The remaining 3 IOIs ranged from 16 to 23 days and were composed of 2 or 3 waves of follicle development (Table 3.1). Hinds with a short first IOI had ovulated for the second time by October 15, and all hinds had ovulated for the second time by October 17. There was a negative correlation between the number of follicles ≥4 mm and the diameter of the dominant follicle ($r = -0.4$, $P < 0.05$). The relationship between the number of follicles ≥4 mm and the diameter profile of the dominant follicle is presented in Fig. 3.1 A.

The CL that developed after the first ovulation was first identified on Day 0 (day of ovulation; n = 13) or on Day 1 (n = 7). In the three hinds that had double ovulations, one had a short IOI (8 Days) with 1 wave of follicle development and maximal CL diameters of 10 and 6 mm, another had a short IOI (12 Days) with two follicular waves and maximal CL diameters of 16 and 13 mm, and the third had a long IOI (23 Days) with 3 waves of follicle development and the 2 CL were juxtaposed and appeared to merge as one structure 19 mm in diameter. The maximum diameter of the CL was smaller during short initial cycles ($P < 0.05$) with one wave of follicle development than in the longer initial cycles

with two or three waves (11.4 ± 0.6, 15.0 ± 1.6 and 19 mm, respectively). Similarly, the number of days that the CL was detected ultrasonographically was fewer ($P < 0.05$) in hinds that had one wave of follicle development than in those with two or three waves (8.5 ± 0.4, 16.0 ± 2.1 and 22 days, respectively). Serum progesterone concentration was weakly correlated with CL diameter ($r = 0.2$, $P = 0.05$; Fig. 3.1 C).

Table 3.1. Dynamics of ovarian follicle development during the first interovulatory interval of the ovulatory season in wapiti.

Number of waves	Interovulatory interval	Follicular wave	Day of wave emergence*	Interwave interval	Maximum diameter of dominant follicle
1 (n=16)	9.1±0.3[a]	1	0.3±0.2	9.1±0.3	11.3±0.2[a]
2 (n=3)	16.3±2.3[b]	1	0.7±0.3	9.0±0.6	12.0±1.0[a]
		2	9.0±0.6	7.3±2.0	9.7±0.7[b]
3 (n=1)	23	1	0	8	13
		2	8	8	12
		3	16	7	12

Mean ± SEM; *Day 0 = ovulation
(a and b) values with different superscripts within a column are different ($P < 0.05$)

Serum FSH concentrations in hinds that had one wave of follicle development during the first IOI fluctuated in a periodic fashion ($P < 0.05$; Fig. 3.1B). Serum FSH concentrations were minimal on Day –3.2 ± 0.4, maximal on Day 0.3 ± 0.4, and minimal again on Day 3.2 ± 0.4. The peak in serum FSH concentration occurred near the trough in follicle numbers (Fig. 3.1B), and on the day the dominant follicle was first detected at 4 mm (i.e., wave emergence).

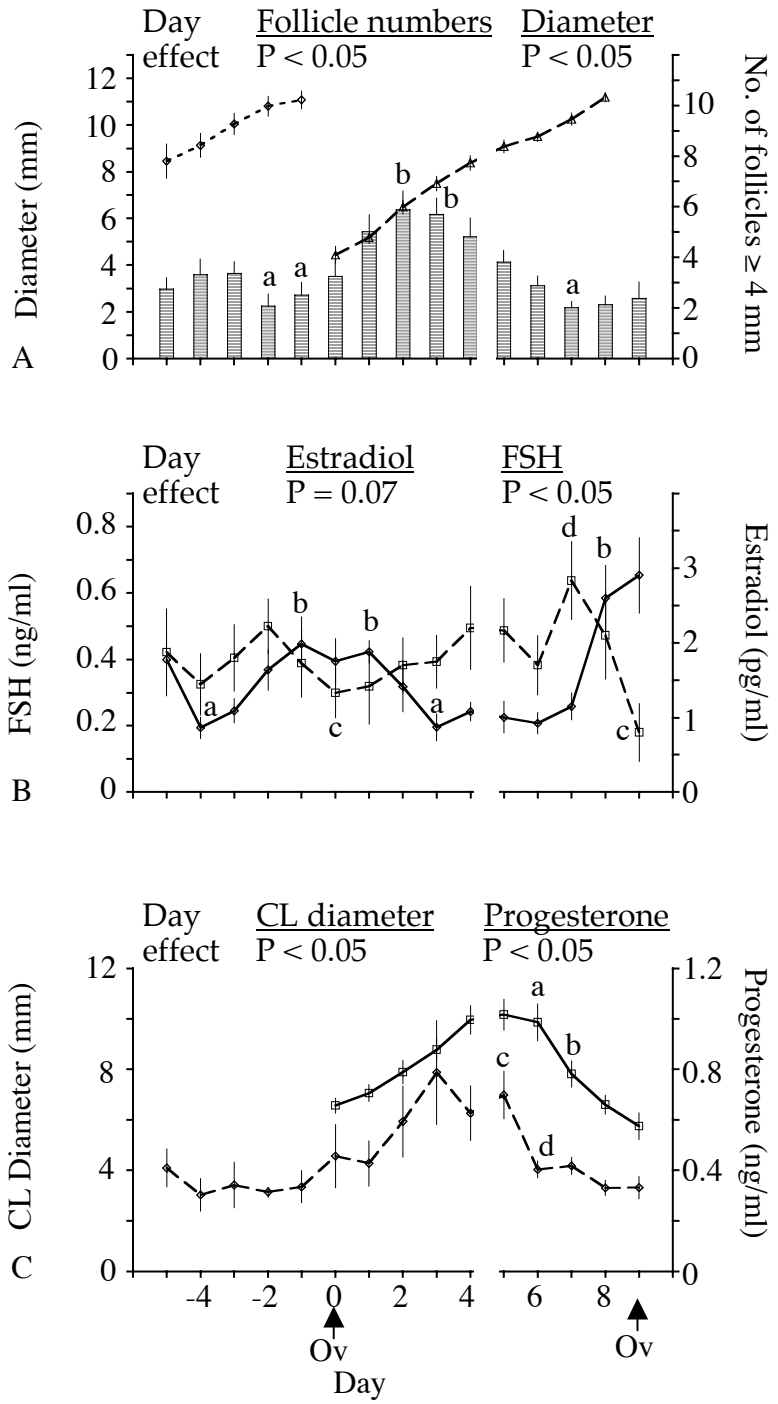

Figure 3.1. Follicle and luteal dynamics (mean ± SEM) in wapiti (n = 15) with one wave of follicle development during the first interovulatory interval: (A) number of follicles ≥ 4 mm in both ovaries (bars) and diameter profiles of the ovulatory follicles (- -), (B) serum concentrations of estradiol (- -) and FSH (—), (C) CL diameter (- -) and serum progesterone concentrations (—).
(a, b, c and d) among days, values with different superscripts are different (P < 0.05)

Serum estradiol concentrations tended to rise and fall in a periodic manner ($P = 0.07$). An apparent rise in estradiol concentration was detected 1 to 2 days before the first and second ovulations of the season (Fig. 3.1B).

3.4.2 Winter transition

Among animals, the last ovulation of the season was recorded on March 22. The distribution of final ovulations is illustrated in Fig. 3.2. The median dates of first and last ovulations of the ovulatory season were Sept. 26 and Feb. 15, respectively, resulting in an ovulatory season of 142 days. The mean duration of the last IOI was 21.2 ± 0.6 days, and was composed of 2 or 3 follicular waves (Table 3.2). The maximum diameter of the last ovulatory follicle (10.0 ± 0.3 mm) was smaller ($P \leq 0.05$) than that of the first or second ovulatory follicles of the fall transition. The number of follicles ≥4 mm detected in both ovaries was negatively correlated with the diameter of the dominant follicle ($r = -0.2$, $P < 0.05$; Fig. 3.3 A).

Serum progesterone concentrations were correlated with CL diameters ($r = 0.7$, $P < 0.05$; Fig. 3.3 C). The maximum diameter of the CL did not differ among the last 3 luteal phases (12.5 ± 0.6 vs 12.0 ± 0.3, 14.4 ± 1.3, and 12.8 ± 0.6 mm, respectively; $P = 0.16$). The maximum diameter of the CL that formed in hinds with 2 follicular waves during the last IOI was smaller than the CL in hinds with 3 follicular waves (12.0 ± 1.3 vs 12.8 ± 0.3 mm, respectively, $P = 0.05$). The CL that formed after the last ovulation was detected for a longer period than the CL of the last interovulatory interval (22.3 ± 1.2 days vs 19.3 ± 0.7, $P < 0.05$).

There were 2, 3, and 4 waves of follicle development during the last luteal period (Table 3.3), and maximum CL diameter did not differ between hinds with 2, 3, or 4 follicular waves (12.0 ± 1.5, 13.0 ± 0.8, and 13.0 ± 1.2 mm respectively). No other luteal structures were observed following the regression of the last CL and anovulatory follicular waves continued to emerge throughout the observational period, which ended in April. No multiple ovulations were detected during the winter transition; only a single CL was observed at any one time in all hinds.

Table 3.2. Dynamics of ovarian follicle development during the last interovulatory interval of the ovulatory season in wapiti.

Number of waves	Interovulatory interval	Follicular wave	Day of wave emergence*	Interwave interval	Maximum diameter of dominant follicle
2 (n=5)	20.4±1.1	1	-0.4±0.2	9.5±0.6^{a}	9.6±0.2^{a}
		2	8.8±0.9	11.0±0.8^{a}	10.8±0.6^{a}
3 (n=6)	21.8±0.3	1	0.0±0.3	7.8±0.4^{b}	10.2±0.2^{a}
		2	7.8±0.4	6.5±0.6^{b}	9.0±0.4^{b}
		3	14.2±0.9	7.5±0.8^{b}	10.0±0.4^{a}

Mean ± SEM; *Day 0 = ovulation
(a and b) values with different superscripts within a column are different ($P < 0.05$)

Serum FSH concentrations fluctuated in a periodic fashion ($P < 0.05$) for > 30 days after the last ovulation (end of the observational period). Peaks in FSH were observed on the day of or the day before follicle wave emergence and the trough in follicle numbers (Fig. 3.3 B). A peak ($P < 0.05$) in serum FSH concentration preceded the emergence of every follicular wave. Regular changes in serum estradiol concentrations were not detected.

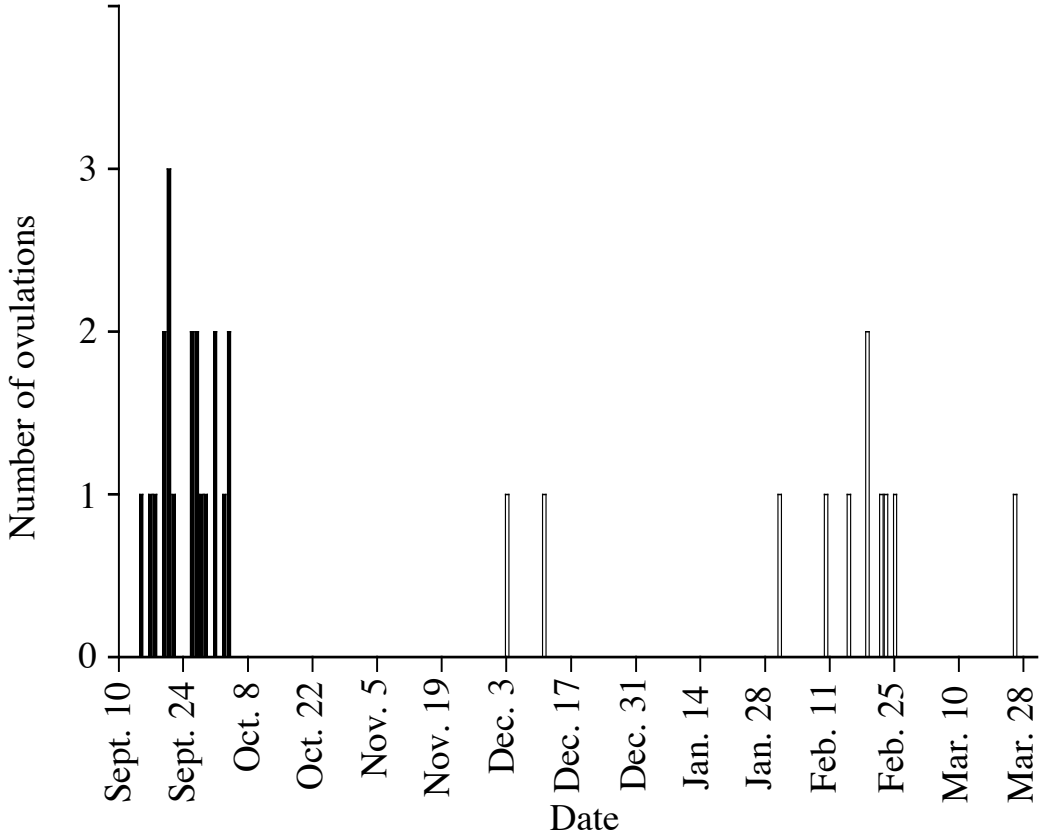

Figure 3.2. The first (solid bars; n=20) and last (open bars; n=11) ovulations of the ovulatory season in wapiti. The median date of the first ovulation was September 26 and the median date of the last ovulation was February 15, which defines a period when ovulations take place of 142 days.

Estradiol serum concentrations at the beginning of the ovulatory season were lower ($P < 0.05$) than those at the end. The mean serum estradiol concentration measured during the last luteal phase of the ovulatory season was 5.6 ± 0.6 pg/ml compared with 1.7 ± 0.6 pg/ml during the first luteal phase. Serum LH concentrations were lower ($P < 0.05$) at the end of the ovulatory season than at the beginning. The mean serum LH concentration during the last luteal phase of the ovulatory season was 0.03 ± 0.01 ng/ml compared with 0.07 ± 0.01 ng/ml during the first luteal phase.

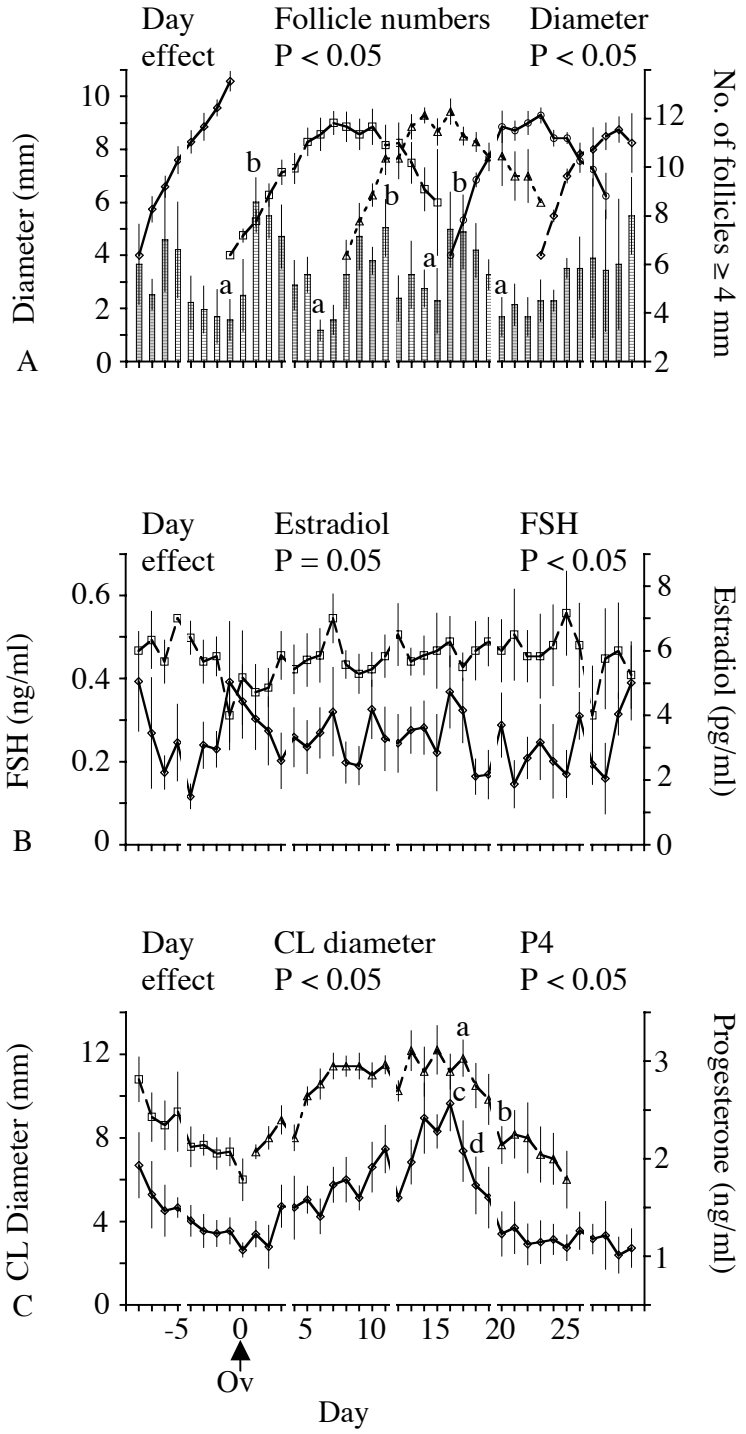

Figure 3.3. Follicle and luteal dynamics (mean ± SEM) in wapiti (n = 7) during the last luteal period of the ovulatory season: (A) number of follicles ≥ 4 mm in both ovaries (bars) and the diameter profiles of successive dominant follicles (- -), (B) serum concentrations of estradiol (- -) and FSH (—), (C) CL diameter (- -) and serum progesterone concentrations (—).
(a, b, c and d) among days, values with different superscripts are different (P < 0.05)

Table 3.3. Dynamics of ovarian follicle development during the last luteal phase of the ovulatory season (period that the CL was detected ultrasonographically), in wapiti.

Luteal phase (days)	Number of waves	Follicular wave	Day of wave emergence	Interwave interval	Maximum dominant follicle diameter (mm)
20.0±3.1	2 (n=3)	1	0.0±0.6	9.3±1.3	10.0±0.6
		2	8.3±0.9	11.3±2.7	9.7±0.9
22.5±1.4	3 (n=4)	1	0.6±0.2	9.2±0.9	9.0±0.5
		2	9.0±1.0	7.0±1.1	8.8±0.7
		3	16.0±0.9	7.3±1.4	9.0±0.5
25.3±1.7	4 (n=4)	1	-0.3±0.5	7.5±0.3	9.8±0.6
		2	7.5±0.3	6.0±0.4	9.8±0.5
		3	13.5±0.5	6.0±0.6	9.5±0.3
		4	19.5±1.0	7.3±1.5	10.0±0.4

Mean ± SEM; Day 0 = ovulation

3.5 Discussion

The ovulatory season, defined by the median dates of first (September 26) and last (February 15) ovulation in each hind, was 142 days. This finding is consistent with results of another study in which the ovulatory season was estimated to be 143 days in wapiti x red deer hybrids based on changing patterns of circulating concentrations of progesterone (Asher *et al*., 2000). The wave pattern of follicle development was maintained throughout both fall and winter transition periods. Follicular wave emergence occurred in a periodic fashion and was invariably preceded by a surge in serum FSH concentration. Unlike previous reports (Glover, 1985), the transition from anovulatory to ovulatory seasons observed in the present study was characterized by a consistent pattern. No luteinized unovulated follicles or other luteal structures were detected before the first ovulation and serum progesterone concentration

remained low until a CL was detected. The first CL of the season was detected only after ovulation, and its ultrasonographic appearance correlated with elevated serum progesterone concentrations. In agreement with findings in several other cervid species (Asher, 1985, Asher *et al.*, 2000, Harder & Moorhead, 1980, Jopson *et al.*, 1990, Thomas & Cowan, 1975), the first IOI of the ovulatory season was shorter (9.1 ± 0.3 days) than the IOI observed during the remainder of the ovulatory season (21.3 ± 0 .1 days; McCorkell *et al.*, 2006)).

The short IOI that characterized the onset of the ovulatory season may be explained by the interaction of steroid hormones on an endometrium that has not been exposed to progesterone for a long period. Estradiol, which is produced by large antral follicles, stimulates the development of oxytocin receptors in the endometrium (Bainbridge *et al.*, 1996). When stimulated, oxytocin receptors cause the release of prostaglandin $F_{2\alpha}$ into the venous flow from the uterus. In ruminants, the ovarian artery lies in close proximity to the venous outflow of the uterus, and prostaglandin diffuses down a concentration gradient from the uterine branch of the ovarian vein to the ovarian artery (Ginther & Del Campo, 1974). Prostaglandin stimulates further release of oxytocin from the CL (Bainbridge *et al.*, 1996, Flint *et al.*, 1994), which in turn, further stimulates endometrial oxytocin receptors and more prostaglandin release. This positive feedback mechanism repeats itself until serum prostaglandin concentrations reach a threshold level that precipitates luteolysis. At the end of the anovulatory season, the endometrium has been conditioned to produce oxytocin receptors by estradiol from repeated waves of antral follicles,

and serum progesterone concentrations have remained low. Exposure of the uterus to progesterone reduces endometrial sensitivity to oxytocin (Beard & Hunter, 1996). Consequently, when the first ovulation takes place and the CL begins to secrete oxytocin, there are a large number of oxytocin receptors available to receive the signal and the result is a large prostaglandin response, which results in early demise of the CL. A similar mechanism has been hypothesized to explain premature luteal regression in superovulated animals (Bainbridge *et al.*, 1996).

An alternative hypothesis to explain the brevity of the first IOI of the ovulatory season is that the granulosa cells of the first ovulatory follicle are less capable of luteinization. Although not critically studied, support for this idea comes from the observations that the interval from emergence to ovulation of the first ovulatory follicle is longer than occurs with subsequent ovulatory follicles (i.e., relatively aged) and the CL subsequent to the first ovulation does not grow as fast or as large, nor does it produce as much progesterone as CL of subsequent cycles in prepubertal bovine calves (Evans *et al.*, 1994). In the present study, however, the intervals from wave emergence to ovulation of the first and second ovulatory follicles were not different (8.5 ± 1.1 and 9.1 ± 0.3 days respectively; $P > 0.05$) nor were their maximum diameters (11.3 ± 1.5 mm vs 11.3 ± 0.9 mm). The capability of conception from the first ovulation of the season has not been critically examined in cervids, but the first ovulation was not associated with estrus or pregnancy in other cervid species (Asher, 1985, Asher *et al.*, 2000, Harder & Moorhead, 1980, Jopson *et al.*, 1990, Thomas & Cowan, 1975). Sexual receptivity and fertility related to the first ovulation may be of

particular interest in wapiti given the predisposition for multiple ovulations in this species at that time.

The observation of multiple ovulations only at the beginning of the ovulatory season may indicate that the mechanism of selection of the dominant follicle is not as efficient as it becomes later in the season. Multiple ovulations were not observed during the ovulatory season (McCorkell *et al.*, 2006) and were not observed during the transition to anovulation. It appears that once the mechanism of selection is established, it continues to function until ovulations cease.

Seasonal changes in LH secretion in seasonally polyestrous animals has been attributed to both steroid-dependent and -independent mechanisms (Meikle & Fisher, 1996) during the anovulatory season that enhance the negative feedback action of estradiol on the secretion of LH (Karsch *et al.*, 1993, Meikle & Fisher, 1996). Compared to the end of the ovulatory season, the fall transition into the ovulatory season in the present study was characterized by higher basal circulating concentrations of LH and lower circulating concentrations of serum estradiol.

One action of LH is to stimulate the production of estradiol from developing antral follicles (Ireland & Roche, 1983). However, in this study estradiol concentrations were higher during the transition from the ovulatory season to the anovulatory season than from the anovulatory season to the ovulatory season, when the opposite might have been expected based on LH

concentrations. Concentrations of estradiol during the ovulatory season were similar to those detected during the transition to the anovulatory season (McCorkell *et al.*, 2006). At the end of the anovulatory season estradiol concentrations were reduced and remained so even as LH concentrations had apparently increased over the anovulatory period. The low estradiol concentration is favorable to higher concentration of LH and therefore ovulation because it effectively removes one mechanism that brings about anovulation, the negative feedback action of estradiol. The higher LH concentrations enhance the growth of the dominant follicle, which enable it to develop to a larger diameter and produce the estradiol needed to stimulate an LH surge that will precipitate ovulation. Despite the apparent reduction in LH concentration during the winter transition, the concentration of estradiol persisted through the winter transition to anovulation. The higher levels of serum estradiol concentration maximized the negative feedback on LH secretion, suppressing serum LH concentrations and inhibiting an LH surge that would cause ovulation. Serum estradiol concentrations lag behind LH concentrations during the transition periods and while this is favorable to the transition-taking place, the mechanism for this phenomenon is unclear.

The maximum diameter of the ovulatory follicles of the first and second ovulations of the ovulatory season were 11.3 ± 1.5 mm and 11.3 ± 0.9 mm, which is similar to the size of the ovulatory follicle in hinds with 2 waves of follicle development during the ovulatory season (11.5 ± 0.3 mm; McCorkell *et al.*, 2006)) and larger than ($P < 0.05$) the ovulatory follicles from the last 2 ovulations of the ovulatory season, 10.1 ± 1.3mm and 10.3 ± 1.1 mm respectively. The

diameters of ovulatory follicles from the last 2 ovulations were similar to the ovulatory follicles in hinds with 3 or 4 follicle waves during the ovulatory season (9.0 ± 0.3 mm; McCorkell *et al.*, 2006)). However, the diameters of ovulatory follicles were larger than the maximum diameter of the dominant follicle reported during the middle of the anovulatory season (7.4 ± 0.2 mm; McCorkell *et al.*, 2004). The anovulatory dominant follicles observed after the last ovulation of the ovulatory season reached a maximum diameter ranging from 8.8 ± 0.7 to 10.0 ± 0.6 mm which appear smaller than the diameters of dominant follicles observed late in the anovulatory season (10.8 ± 0.2 mm; McCorkell *et al.*, 2004)).

The average length of the last IOI was not different ($P > 0.05$) from the preceding 2 IOI (21.2 ± 0.6 days vs 21.3 ± 0.4 days and 21.3 ± 05 days respectively) and was similar to the IOI reported during the ovulatory season of 21.3 ± 0.1 days (McCorkell *et al.*, 2006). It has been reported in fallow deer that the IOI gets progressively longer as the anovulatory season approaches (Asher, 1985). While the IOI did not become progressively longer in this study, the maximum diameter of the first dominant follicle of hinds with 2 follicle waves appears smaller at the end of the ovulatory season than the reported diameter during the ovulatory season (9.6 ± 0.2 vs 12.5 ± 0.3 (McCorkell *et al.*, 2006)). The reduction in the size of the first dominant follicle may lead to a shorter IWI and the increased occurrence of 3 and 4 follicular waves during the IOI. The IOI of 3- and 4-wave animals is longer than that observed for 2-wave animals. Therefore, if an increasing number of animals converted to 3- and 4-wave patterns as the anovulatory season approached, the result would be an apparent increase in

length of the average estrous cycle. In this study the ratio of 2-wave to and 3-wave animals did not change from the approximately equal numbers reported during the ovulatory season (McCorkell *et al.*, 2006). However, small changes in the length of the IOI may be found with a larger sample size.

The transition from the ovulatory season to the anovulatory season occurred over a wide interval with the first hind entering the anovulatory season in January and the last in April. This is in agreement with previously published observations (Glover, 1985, Guinness *et al.*, 1971) but in contrast to the transition from anovulatory season to the ovulatory season, which took place over a 3-week period beginning in the middle of September. In this study, unlike previous reports (Glover, 1985) irregular cycle lengths or CL development did not mark the transition to the anovulatory season. Instead, the IOI remained constant, as did the development of follicular waves. The only change was the failure of the dominant follicle to ovulate once the CL had regressed.

In conclusion, estrous cycles during transition into the ovulatory season have been described as being irregular and those out of the ovulatory season as increasingly long. In the present study, the transition periods at the beginning and end of the ovulatory season were characterized by regular events based on the consistent underlying process of the wave-like development of ovarian follicles. The median dates of the first and last ovulations of the ovulatory season were September 26 and February 15, respectively, which defined an ovulatory season of 142 days. Transition to regular estrous cycles was preceded by one short IOI (9 days) characterized by 1 follicular wave and a small, short-

lived and hypo-functional CL. Multiple ovulations were observed only during the transition to regular estrous cycles in 20% of the animals. The last IOI of the ovulatory season was similar to that reported during the rut (21 days). Transition to anovulation was marked by a failure of the dominant follicle to ovulate after luteal regression.

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4.0 EVALUATION OF AN OVARIAN SYNCHRONIZATION SCHEME FOR FIXED-TIME ARTIFICIAL INSEMINATION IN WAPITI.

4.1 Abstract

The objective was to characterize ovarian follicle development in wapiti subsequent to an empirically-derived treatment protocol used commercially for fixed-time insemination. Wapiti hinds were examined daily by transrectal ultrasonography from September 14 to November 1 (the period of transition into the ovulatory season). On September 29 (Day 0) hinds were assigned to a treatment group (n = 7) and given an intravaginal progesterone-releasing device (CIDR-B, 1.9 g of progesterone), or an untreated control group (n = 9). On Day 14 the CIDR was removed and 200 IU eCG was given im to hinds in the treatment group. Blood samples were collected every second day to measure serum progesterone concentrations. Hinds in the control group ovulated randomly over a 15-day period beginning September 29. In the treatment group, 5 hinds ovulated 3 days after eCG treatment (October 16), 1 ovulated 7 days after treatment (October 20), and 1 failed to ovulate by November 1. All extant dominant follicles ceased growth and/or began to regress within 2 days of CIDR placement. Two waves of follicular development were detected between the times of CIDR insertion and removal in the treatment group; the first emerged 5.1 ± 0.5 days after CIDR insertion and the second at 11.0 ± 0.7 days after insertion. Emergence of the first follicular wave in the control group

ranged from Days 1 to 9 and was more variable than in the treatment group ($P \leq 0.05$). Serum progesterone concentration was 0.6 ± 0.5 ng/ml before CIDR placement, 6.1 ± 1.4 ng/ml the day after placement, and maximal 3 days after placement at 8.8 ± 1.8 ng/ml; maximal luteal-phase progesterone concentration (1.1 ± 0.1 ng/ml) was lower in the control group ($P < 0.05$). Progesterone concentration was 6.1 ± 1.1 ng/ml the day before CIDR removal and fell to 0.8 ± 0.9 the day after. The protocol to synchronize ovulation was effective in 5/7 (71%) hinds, and 4 of 7 (57%) became pregnant. In conclusion, synchronization with CIDR-B was effective, but the duration of CIDR-B placement was unnecessarily long. The protocol may be improved by shortening the interval of CIDR placement and by reducing the circulating concentrations of progesterone to physiologic levels (<4 ng/ml).

4.2 Introduction

An empirically designed treatment protocol to synchronize estrus has been used to facilitate artificial insemination in red deer and wapiti (Fennessy *et al.*, 1989). The synchronization protocol was first developed in sheep (Robinson, 1965) under the notion that luteal phase progesterone prevents final follicular maturation in an otherwise continuous pattern of follicle growth; i.e., that an ovulatory follicle may be selected from an ever-ready pool based on the coincidence of its maturity and the onset of luteolysis (Hafez, 1980). Hence, synchronous withdrawal of an artificially prolonged luteal phase was used in an effort to induce a consistent interval to ovulation and synchrony among animals.

Under this paradigm, initial synchronization protocols in sheep used repeated injections of progesterone followed by a single injection of eCG. In an early study, sheep treated with progesterone for the longest period (15 days) had the highest percentage of ewes that came into estrus (Dutt, 1952). Further refinement of the protocol led to the development of methods to deliver progesterone intravaginally (Robinson, 1965) and the general acceptance that eCG should be given following withdrawal of progesterone (Gordon & Keane, 1967).

A nearly identical protocol has been used in red deer and wapiti and involves the use of a controlled intravaginal drug-releasing device (CIDR) that delivers a continuous concentration of progesterone, and eCG upon CIDR withdrawal

(Fennessy *et al.*, 1989). The CIDR device is most commonly inserted for 12 to14 days (Asher *et al.*, 2000, Bowen, 1989, Fennessy *et al.*, 1990, Fisher *et al.*, 1986). Longer insertion periods have been associated with lower conception rates in red deer (Fennessy *et al.*, 1990). It has become routine to administer 200 to 250 IU of eCG at or near the time of CIDR withdrawal (Asher *et al.*, 2000, Fennessy *et al.*, 1989). Three reasons have been cited for this practice (Asher *et al.*, 1993): 1) an increased incidence of ovulation compared to progesterone treatment alone (Fennessy *et al.*, 1989, Fisher *et al.*, 1986), 2) extra gonadotrophic stimulation may be necessary to overcome the effects of stress (Fennessy *et al.*, 1989), and 3) eCG treatment may increase the synchrony of ovulation in a group of hinds (Fennessy *et al.*, 1989).

Recent research, however, indicates that ovarian follicle development in wapiti occurs in a wave-like pattern (McCorkell chapter 3, McCorkell *et al.*, 2006, McCorkell *et al.*, 2004); i.e., ovarian follicles are not at a constant state of readiness within or among estrous cycles. A wave of follicle development is characterized by the synchronous development of a group of small follicles that grow in diameter at an equal rate until one follicle is selected to continue growing while the rest regress. The selected follicle is functionally dominant over the subordinate follicles and is responsible for the cessation of growth and ultimate regression of the subordinate follicles. The growing dominant follicle present at the time of CL regression will then go on to ovulate (Adams *et al.*, 1993, Ginther *et al.*, 1989, Ko *et al.*, 1991, Pierson & Ginther, 1988). Most estrous cycles in wapiti were composed of 2 or 3 waves of follicle development (McCorkell *et al.*, 2006).

In cattle, progesterone has also been used to synchronize estrus and it has been shown that progestins given for a period longer than the CL life span (> 14 days) result in synchronous estrus upon withdrawal, but with reduced fertility (Kinder *et al.*, 1996). Reduced fertility is thought to be the result of aged oocytes from "persistent" follicles (Revah & Butler, 1996). Given that synchronization protocols are initiated without regard to where the individual animals are in their cycle of ovarian follicle development, the risk of a prolonged period of progesterone exposure (i.e. longer than the normal CL lifespan) is high, and reduced fertility may be expected. Despite this, high pregnancy rates in wapiti (~70%) have been reported with the previously described estrous synchronization protocol (DeGrofft, 2000).

The concept that ovarian follicle development in wapiti is not continuous but rather occurs in waves provided impetus to critically examine the effects of an empirical progesterone-based protocol originally designed to synchronize estrus in sheep and cattle. The objective of this study was to characterize ovarian follicle development and ovulation synchrony in wapiti subsequent to an empirically derived treatment protocol used commercially for fixed-time insemination.

4.3 Material and methods

An ovarian synchronization scheme was tested in a group of 16 wapiti hinds, aged 2 to 14 years, maintained on a farm near Saskatoon, Saskatchewan (52°07′N, 106°38′W). The hinds were fed alfalfa/brome hay in a 1-hectare pen that was separated from the stag's pen by 2 fences 7 meters apart. The hinds were moved daily from the pen through a 70 meter-long alley to the handling facility and restrained in a squeeze chute for treatment and examination (McCorkell *et al.*, 2001).

The hinds were assigned to a treatment group (n = 7) or an untreated control group (n = 9), and examined daily by transrectal ultrasonography from September 14 to November 1 – the period of transition into the ovulatory season. On September 29 (Day 0), hinds in the treatment group were given an intravaginal progesterone-releasing device (CIDR-B, 1.9 g progesterone; Bioniche Animal Health Inc., Belleville, Ontario, Canada). On Day 14, the CIDR was removed and 200 IU eCG (Pregnecol, Bioniche Animal Health Inc., Belleville, Ontario, Canada) was given intramuscularly.

The ovaries were examined daily by transrectal ultrasonography (Aloka SSD500, Instruments for Science and Medicine, Vancouver, Canada) using a 7.5 MHz linear-array transducer. A sketch of each ovary was made during the examination detailing the size and location of follicles ≥4 mm in diameter and the corpus luteum. The drawings were used to tabulate the number of follicles ≥4 mm within the pair of ovaries of each hind for each day of the observational

period and to construct diameter profiles of individually identified follicles from first detection at 4 mm until they could no longer be individually identified (regressed to ≤4 mm) (Knopf *et al.*, 1989, McCorkell *et al.*, 2004). Ovulation was defined as having occurred if a follicle ≥8 mm in diameter identified during the previous day's examination was not present on the subsequent day, and a corpus luteum (CL) was identified at the same location within the next 3 days.

Hinds in the treatment group were artificially inseminated once only, at 65 hours after CIDR withdrawal (DeGrofft, 2000). Hinds in the control group were artificially inseminated once only, at presumptive estrus based on ultrasonographic evidence of a growing large dominant follicle (>8 mm), a regressing CL, and fluid accumulation in the cranial portion of the vagina. Cryo-preserved semen from a single stag was used for all inseminations. Transrectal ultrasonography at 30 to 40 days after insemination was used to diagnose pregnancy (Bingham *et al.*, 1990, Revol & Wilson, 1991). Calving rate was recorded the following summer.

4.3.1 Blood samples and hormone assays

Blood samples were collected in 10 ml vials without anticoagulant every second day from 8 animals in the control group and 5 in the treatment group (3 hinds were not amenable to blood collection) via jugular venipuncture using an 18-gauge 3.8 cm needle. Blood samples were kept chilled until centrifugation (<3 hours) at 1500g for 10 minutes. The serum was removed and stored at -20°C.

Serum progesterone concentration was measured using a chemiluminescence assay previously validated in wapiti (Immulyte, Diagnostic Products Corporation, Los Angeles, California; (McCorkell *et al.*, 2006)). Intra-assay coefficients of variation ranged from 4.9% to 13.5% for the low reference sample and 3.0% to 12.0% for the high reference sample. Over 10 assays, the interassay coefficients of variation were 14.7% and 5.5% for the low and high reference samples, respectively.

4.3.2 Data analysis

A wave of follicle development was defined as the synchronous growth of a group of small follicles. The dominant follicle was defined as a follicle that attained a diameter ≥8 mm and exceeded the diameter of all others. Follicle selection was defined as the day the dominant follicle became and remained larger than any of its cohorts. The day the dominant follicle of a wave was first detected at 4 mm in diameter was defined as the day of wave emergence.

For the purposes of analysis and illustration, data for day-to-day profiles were centralized to the day of wave emergence and divided into intervals encompassing successive wave emergence. For the last follicular wave, data included several days before ovulation. For all other waves, data included an interval of several days before and after the day of wave emergence (indicated by broken lines in Fig. 4.2).

All data are presented as the mean ± standard error of the mean (SEM). Serial data were examined by analysis of variance for repeated measures using the PROC MIXED procedure of the SAS System (SAS System 8, Cary, N.C.). The degree of variability in the interval from treatment to wave emergence or to ovulation was estimated by calculating the group mean and subtracting it from each data point; the resulting values were then compared between groups by student's t-test (Ratto *et al.*, 2003). Non-serial data were compared between treatment and control groups by student's t-test.

4.4 Results

The CIDR device did not interfere with transrectal ultrasonographic examinations, and was retained by all hinds until removal on Day 14. However, vaginal inflammation was apparent by accumulation of fluid in the vagina, detected ultrasonographically in 3 hinds beginning 2 days after placement of the device, and by swelling and edema adjacent to the tips of the wings of the CIDR device in 4 hinds beginning 9 to 12 days after placement.

No CL were detected at the beginning of the observational period (September 14), but spontaneous ovulations were detected before the time of CIDR placement (Day 0, September 29) in 2 of 7 hinds in the treatment group and in 8 of 9 hinds in the control group. In the control group, 7 hinds ovulated for the second time within 12 days of their first ovulation (interovulatory interval, 9.9 ± 0.5 days), while the remaining 2 hinds ovulated 20 and 23 days later.

Beginning on Day 0, ovulations in the control group occurred randomly over a 15-day period (Fig. 4.1). In the treatment group, 5 hinds (including the 2 that had ovulated prior to CIDR placement) ovulated 3 days after eCG treatment (October 16), 1 ovulated 7 days after treatment (October 20), and 1 failed to ovulate by November 1 (end of the observational period).

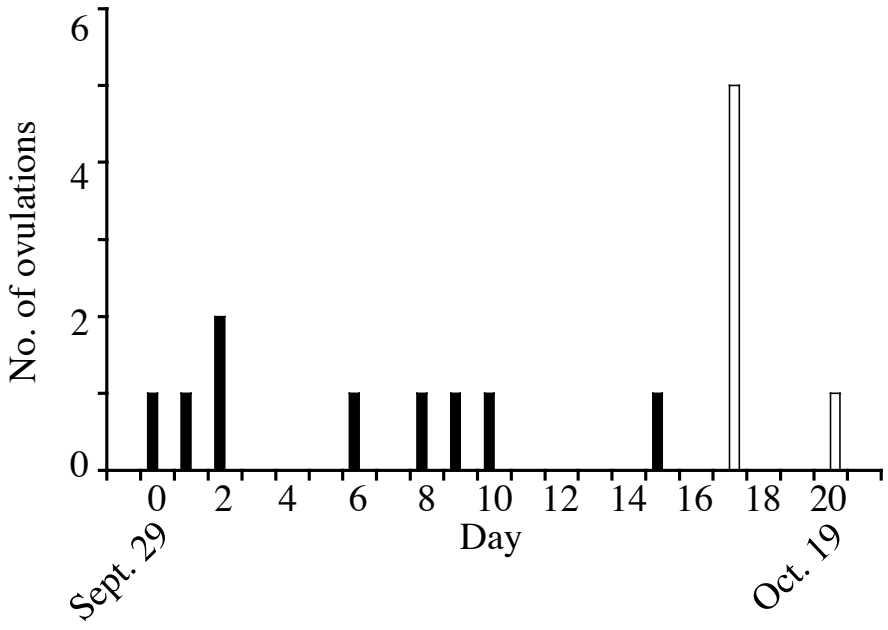

Figure 4.1. Distribution of ovulation in wapiti given an ovarian synchronization treatment (CIDR and eCG; n=7, open bars) at the beginning of the breeding season and in untreated controls (n=9, solid bars). Day 0 is the day of CIDR device insertion in the treatment group (September 29).

All extant dominant follicles ceased growth or began to regress within 2 days of CIDR placement (Fig. 4.2). The mean interval from initiation of treatment (Day 0) to emergence of the first wave was not different between groups, but the variation in the interval was greater ($P < 0.05$) in the control group than in the treatment group (Table 4.1). The mean interval to ovulation was greater ($P < 0.05$) in the treated group, but was more variable ($P < 0.05$) in the control

group (Table 4.1). Two waves of follicle development were detected between the time of CIDR insertion and CIDR removal in 6 treated hinds, and the ovulatory follicle was of the second wave after Day 0 in 6 of 7 treated hinds versus the first wave in 7 of 9 untreated control hinds (Table 4.1). The second follicular wave emerged 11.0 ± 0.7 days (range: Day 9 to Day 14) after CIDR insertion in 6 treated hinds; the remaining hind in the treatment group had only one wave of follicle development and the dominant follicle persisted until Day 21 when it ovulated (7 days after CIDR withdrawal) at a diameter of 18 mm. The maximum diameter of the dominant follicle of the first wave after CIDR insertion was 9.2 ± 0.3 mm (n = 6) and the maximum diameter of the ovulatory follicle was 11.0 ± 0.6 mm (n = 5). The mean diameter of the ovulatory follicle in the control group, measured during the treatment period, was 11.0 ± 0.3 mm (n = 9; Table 1). Only 1 hind (treated animal) failed to ovulate during the study; wave emergence was detected on Day 8 and again on Day 14, and maximum follicle diameters for the 2 waves were 8 mm and 13 mm, respectively.

Serum progesterone concentrations in the treatment group were higher ($P < 0.05$) than in the control group throughout the observational period (Fig. 4.3). Concentrations were not different between treatment and control groups before the time of CIDR placement (0.6 ± 0.2 ng/ml and 0.7 ± 0.1 ng/ml, respectively), but increased ($P < 0.05$) in the CIDR treated group to 6.1 ± 1.6 ng/ml the day after placement, and were maximal 3 days after placement at 8.8 ± 2.3 ng/ml. Maximal luteal-phase progesterone concentration in the control group (1.1 ± 0.1 ng/ml) was lower than the serum progesterone concentrations measured in the treatment group ($P < 0.05$). In the treatment group, the serum

progesterone concentration was 6.1 ± 1.2 ng/ml the day before CIDR removal and fell to 0.8 ± 0.4 ng/ml the day after removal (Fig. 4.3).

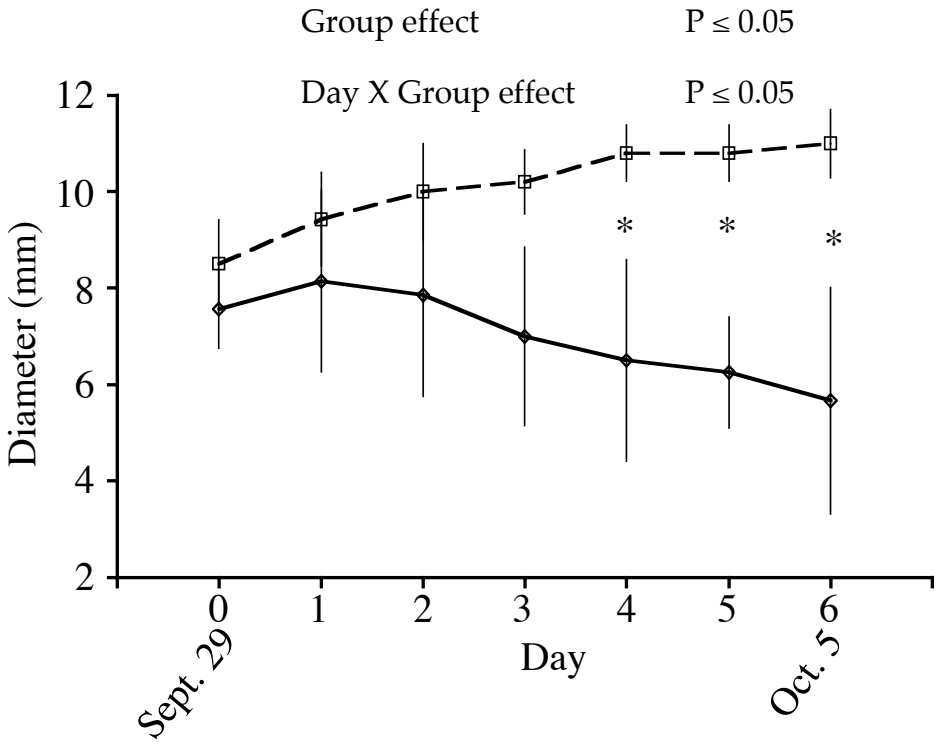

Figure 4.2. The mean diameter profile of the dominant follicle present at the time of CIDR device insertion for each hind in the treatment (◇)and control (□) group. Day 0 is the day of CIDR insertion. Mean ± SEM
* indicates values are different P < 0.05.

Pregnancy rates and calving rates were similar between groups (Table 4.1). The 4 treated hinds that became pregnant ovulated on Day 17 (3 days after CIDR removal and eCG treatment, and 1 day after insemination).

Table 4.1. Response of wapiti hinds following an ovarian synchronization treatment (CIDR and eCG) and fixed-time artificial insemination.

	Treatment Group (n=7)	Control Group (n=9)
Interval (days) from Day 0 to 1st wave emergence (range)	5.1 ± 0.5 [a] (4 -8)[a]	4.6 ± 1.1 [a] (0 -9)[b]
Interval (days) from Day 0 to ovulation* (range)	17.5 ± 0.5 [a] (17 - 21)[a]	4.9 ± 1.7 [b] (0 -15)[b]
Max. diam. of 1st dominant follicle after Day 0 (mm) (range)	10.4 ± 1.3 (8 - 18)	12
Max. diam. of 1st ovulatory follicle after Day 0 (mm)* (range)	12.2 ± 1.3 [a] (10 - 18)[a]	11.0 ± 0.3 [a] (9 - 12)[a]
Number of waves between Day 0 and ovulation	1.9 ± 0.1[a]	1.3 ± 0.2[b]
Ovulation rate**	5/7[a]	9/9 [a]
Pregnancy rate	4/7 [a]	6/9 [a]
Calving rate	4/7 [a]	5/9 [a]

Mean ± SEM; Day 0 = CIDR placement (September 29)
* n=6 in the treatment group (hinds that ovulated)
**In the treatment group, only synchronous ovulations (i.e., on same day)
(a and b) Between groups, means with no common superscript differed (P<0.05).
(x and y) Between groups, variances with no common superscript differed (P<0.05).

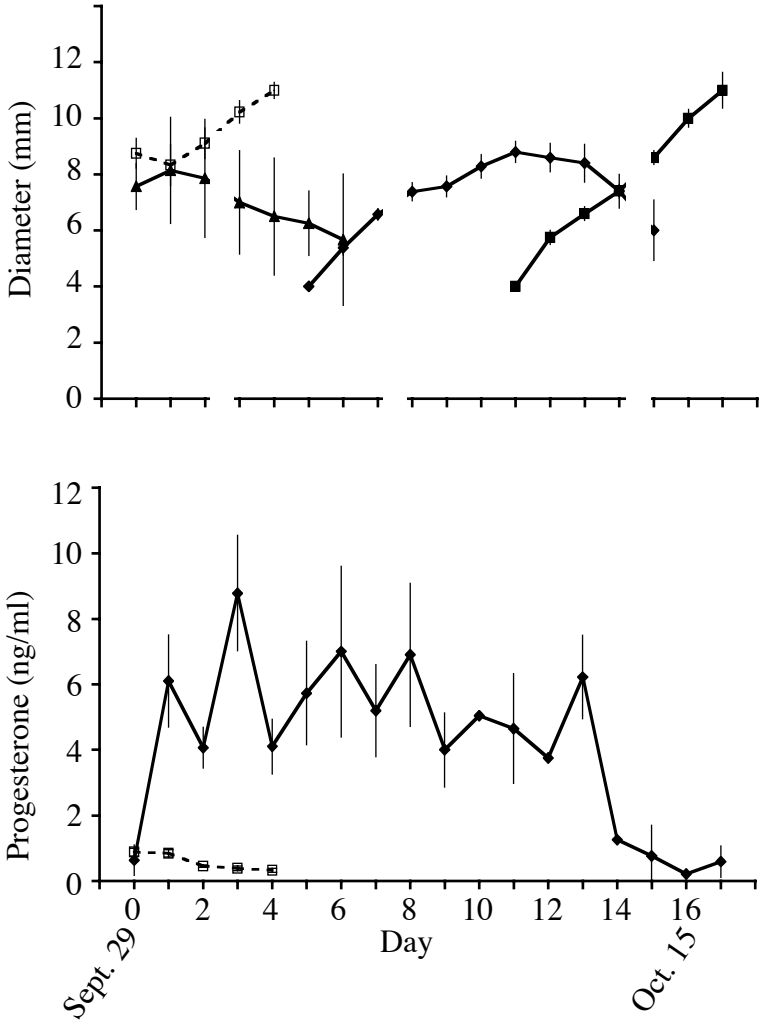

Figure 4.3. The diameter profiles of the dominant follicles and the serum progesterone concentrations from the treatment (solid line) and control (dashed line) groups after CIDR insertion on Day 0. The CIDR was removed on Day 14.

4.5 Discussion

The ovulations that were recorded before the treatment period were the first ovulations of the breeding season and were, in most cases, followed by a short-lived CL, which is consistent with previous observations (McCorkell, chapter 3). Although it has not been examined critically in wapiti, the short first IOI of the season is probably not fertile because the short luteal phase with low levels of progesterone would not allow enough time for maternal recognition of pregnancy. In contrast, the second ovulation of season is fertile as evinced in the control group where 7 of the 9 hinds became pregnant after insemination at the second ovulation of the season.

Five of the 7 hinds in the treatment group did not ovulate prior to CIDR placement. Three of the 5 ovulated synchronously and all 3 were subsequently found to be pregnant. Hence, these animals became pregnant after their first ovulation of the breeding season – an ovulation that was preceded by exogenous progesterone. The CIDR device in treated hinds appeared to have a similar effect as the short-lived CL in untreated hinds; a period of progesterone exposure prior to ovulation is requisite for establishment of pregnancy.

The treatment protocol was successful in synchronizing ovulation in 5 of 7 (71%) hinds, leading to a pregnancy rate of 4 of 7 (57%) inseminated or 4 of 5 (80%) that ovulated near the time of insemination. One hind in the treatment group developed a dominant follicle that persisted for a longer period and grew to a greater diameter than any other dominant follicle. In cattle, oocytes

ovulated from persistent, oversized follicles were less fertile (Revah & Butler, 1996).

Synchronous emergence of follicular waves 5 days after CIDR insertion was an unexpected finding in the present study. In cattle, wave emergence was not synchronized by the use of CIDR devices alone (Martinez *et al.*, 2000). When only a CIDR device was used, follicular waves emerged over a 10-day period and the diameter of the dominant follicle present at the time of CIDR insertion did not change for the ≥5 days post insertion (Martinez *et al.*, 2000). A study in llamas observed that regardless of the stage of follicular development at the time of CIDR insertion the diameter of the dominant follicle began to decline (Chaves *et al.*, 2002) and reached a minimum diameter 7 days after treatment. This finding is similar to what was observed in the present study, where all extant dominant follicles ceased growth or began to regress within 2 days of CIDR placement.

The disparate effects between cattle and wapiti studies may be related to the concentration of serum progesterone that was attained after CIDR placement. Plasma progesterone concentrations in cattle treated with CIDR devices were not different from mid-diestrus plasma progesterone concentrations in untreated control animals (5 to 7 ng/ml) (Macmillan *et al.*, 1991). In the present study, however, CIDR-treated hinds had serum progesterone concentrations that were 6 times higher than in untreated hinds, and 2 times higher than reported mid-diestrus concentrations measured during the breeding season (McCorkell *et al.*, 2006, chapter 3). Moreover, high progesterone concentrations

were maintained throughout the treatment period in wapiti, unlike cattle in which levels dropped 3 days after CIDR insertion (Macmillan *et al.*, 1991). It is noteworthy that the CIDR devices used in the present study contained a similar amount of progesterone (1.9 g), to the type used in cattle (Martinez *et al.*, 2000) but a greater amount than CIDR devices used in previous work in red deer and llamas (0.365g and .33g respectively of progesterone; Asher *et al.*, 1993, Fennessy *et al.*, 1989, Chaves *et al.*, 2002).

The synchronous emergence of the first follicular wave 5 days after CIDR insertion was unexpected and may be attributed to the supraphysiologic concentrations of progesterone attained in this study. In cattle, treatment with progesterone alone was not associated with a predictable interval to new wave emergence (Martinez *et al.*, 2000), and although progesterone suppressed the growing phase of the dominant follicle in a dose-dependent manner (Adams *et al.*, 1992a) periodic emergence of follicular waves continued, even when serum progesterone concentrations were twice physiological levels. Circulating FSH concentrations were not directly affected by progesterone treatment (Adams *et al.*, 1992a), but were indirectly influenced by the suppressive effect of progesterone on the duration of follicular dominance. It appears that high serum progesterone concentrations in wapiti during the seasonal transition to the breeding season was profoundly suppressive to follicular growth such that follicular dominance was removed, and serum FSH was allowed to surge resulting in new wave emergence (Adams *et al.*, 1992b, McCorkell *et al.*, 2006, chapter 3). Emergence of the second follicular wave after CIDR insertion was more variable than the first; therefore, ovulation of the dominant follicle of the

first wave may result in better synchrony than that of the second wave (i.e., remove the CIDR on Day 6 or 7).

The CIDR device was retained by all of the hinds in the treatment group but evidence of inflammation in the vagina was noted. Fluid accumulated in the vagina as early as 2 days after insertion, and swelling and edema associated with the wing tips of the CIDR device was noted between 9 and 12 days after insertion. Although local vaginal inflammation did not appear to affect fertility (4 of the 5 hinds that ovulated synchronously became pregnant), reducing the length of the wings of the CIDR device may reduce vaginal swelling and edema and may also be helpful in reducing the high serum progesterone concentrations found in this study.

In conclusion, the CIDR-based protocol resulted in synchronous ovulation in 5 of 7 (71%) hinds, 4 of which became pregnant after fixed-time insemination with cryo-preserved semen. The treatment period encompassed 2 waves of follicle development, the first of which emerged synchronously on Day 5. Reducing the duration of CIDR placement to 6 - 7 days may increase the number of animals that ovulate synchronously. One hind developed a persistent over-sized follicle.

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5.0 INDUCTION OF OVARIAN FOLLICULAR WAVE EMERGENCE IN WAPITI (*Cervus elaphus*)

5.1 Abstract

Two experiments were conducted during the late anestrous period (July and August) to test the effects of treatments designed to electively induce ovarian follicular wave emergence for the purposes of group synchronization. In Experiment 1, hinds were randomly assigned to 3 groups and given saline im (Controls; n = 5), 5 mg of estadiol-17β im (n = 4), or 5 mg estradiol-17β plus 100 mg progesterone im (n = 5). In Experiment 2, hinds were divided into 2 groups and given a no treatment (Controls; n = 6), or underwent transvaginal ultrasound-guided follicle ablation (n = 7). In both experiments, ovarian follicular dynamics were monitored by daily transrectal ultrasonography from Day 0 (day of treatment) to Day 8. In Experiment 1, blood samples were collected at each examination for measurement of serum progesterone and FSH concentrations. The mean day of wave emergence did not differ between the control and estradiol alone groups, but tended to be later in the estradiol plus progesterone group (Day 4.0 ± 0.7, Day 3.5 ± 0.3, Day 5.2 ± 0.2, respectively; P = 0.08). Compared to the control group, the interval from treatment to wave emergence was less variable in the estradiol-17β alone group (P = 0.07) and the estradiol-17β plus progesterone group (P < 0.05). The day of wave emergence was more variable (P < 0.05) and tended to be later (P = 0.10) in the

control group compared to the ablation group (Day 2.5 ± 0.8 versus Day 1.4 ± 0.2). All 3 treatments tested were successful in synchronizing ovarian follicular wave emergence among a group of wapiti hinds and may be useful for estrus synchronization and superstimulatory protocols.

5.2 Introduction

To facilitate the application of assisted reproductive technologies in wapiti and red deer, attempts have been made to synchronize ovarian function among animals through the use of various hormones, either alone or in combination (Asher *et al.*, 1994, 1995, 1993, Fennessy *et al.*, 1989, Fisher *et al.*, 1994, 1989, Haigh *et al.*, 1988, Jabbour *et al.*, 1991, 1994, Scott *et al.*, 2000). The focus of these treatments has been to control the luteal phase of the estrous cycle by either prolonging it with exogenous progestogens or shortening it with prostaglandins. The status of ovarian follicle development at the time of synchronization treatments, however, was not taken into account in past studies. In cattle, follicular status was a significant source of variability in response to synchronization treatments involving manipulation of the progestational phase (Adams, 1994, Huhtinen *et al.*, 1992, Kastelic *et al.*,1991, 1990, Nasser *et al.*, 1993, Pierson & Ginther, 1988, Roche & Prendiville, 1979, Savio *et al.*, 1990).

Transrectal ultrasonography has provided a method of critically monitoring ovarian follicle development in wapiti (McCorkell *et al.*, 2001), and has resulted in the recent discovery and characterization of ovarian follicular wave development during both the breeding (McCorkell *et al.*, 2006) and non-breeding seasons (McCorkell *et al.*, 2004). Similar to the pattern described for cattle (Ginther *et al.*, 1989a, 1989b), most estrous cycles in wapiti (85%) were composed of 2 or 3 follicular waves, each of which was characterized by the simultaneous growth of a group of follicles followed by selection of a single

dominant follicle for continued growth and subsequent regression of the remaining subordinate follicles In cattle, the dominant follicle of the wave is responsible for the suppression of subordinate follicles and for suppression of new wave emergence (Adams *et al.*, 1993a, 1993b, Ko *et al.*, 1991). Therefore, strategies have been developed to remove the suppressive effects of the dominant follicle in an effort to electively induce wave emergence and improve ovarian synchronization among animals for the purposes of controlled breeding.

Two strategies have been used in cattle to remove follicular dominance and electively induce wave emergence, hormonal treatment and physical ablation. In an early study, administration of estradiol-17β was associated with cessation of growth and early onset of regression of the dominant follicle of a wave (Bo *et al.*, 1994). The effect was more consistent when combined with progesterone treatment; a new wave of follicle development emerged consistently 4 to 5 days after treatment of cows with 2 to 5 mg estradiol-17β and 50 to 100 mg of progesterone (reviewed in Adams, 1998, Bo *et al.*, 1995a). This treatment has been used successfully in cattle in protocols for fixed-time insemination (Martinez *et al.*, 2000) and ovarian superstimulation (Mapletoft *et al.*, 2002).

The second strategy for controlling wave emergence in cattle involved physical destruction of all large antral follicles. In early studies, surgical electro-cautery of the dominant follicle was found to hasten the emergence of the next follicular wave (Adams *et al.*, 1993a, Ko *et al.*, 1991). Subsequent studies revealed that

transvaginal ultrasound-guided follicle aspiration of all follicles ≥5 mm in diameter (Bergfelt *et al.*, 1994) or just the largest follicle in each ovary (Baracaldo *et al.*, 2000) was effective in eliciting follicular wave emergence 1 to 2 days later.

The objective of the present study was to determine if follicular wave emergence could be electively induced in wapiti using techniques developed previously for use in cattle. We tested the hypothesis that hormonal treatment (estradiol with or without progesterone; Experiment 1) or physical ablation of ovarian follicles (transvaginal ultrasound-guided follicle aspiration; Experiment 2) will result in follicular wave emergence at a consistent interval post-treatment in wapiti.

5.3 Materials and methods

5.3.1 Experiment 1

Wapiti hinds (n = 14), 2 or 3 years of age and weighing between 168 to 214 kg, were used during the late anestrous period (July 13 to 26). The hinds were maintained on alfalfa pasture in a 4-hectare pen on a farm near Saskatoon, Saskatchewan (52°07′N, 106°38′W). The hinds were moved daily from the pen through a 300 meter-long alley to the handling facility for examination. In the handling facility, the hinds were restrained in a squeeze chute where transrectal ultrasonography was done. Daily ultrasonographic examinations were initiated on July 13 using a 7.5 MHz transducer (Aloka SSD-500, Instruments for Science & Medicine, Vancouver, BC; (McCorkell *et al.*, 2004) to document ovarian follicular status prior to treatment. During examination, drawings of each ovary

were made to record the size and location of all follicles ≥4 mm in diameter and the corpus luteum (Knopf *et al.*, 1989, McCorkell *et al.*, 2004). On July 17 (Day 0), the hinds were assigned randomly to one of three treatment groups and given 2 ml saline im (control group; n = 5), 5 mg of estradiol-17β in 2 ml of canola oil im (Bo *et al.*, 1995a; n = 4), or 5 mg estradiol-17β plus 100 mg progesterone in 2 ml of canola oil im (n = 5). The ovarian follicular response to treatment was monitored daily by transrectal ultrasonography until Day 9 (July 26).

5.3.1.1 Blood samples and hormone assays

A blood sample was collected at each examination via jugular venipuncture using an 18-gauge 3.8 cm needle into a vial without anticoagulant. Blood samples were kept chilled for a period of ≤3 hours until centrifugation at 1500g for 10 minutes. The serum was removed and stored at -20°C.

Serum progesterone concentration was measured using a chemiluminescence assay previously validated for use in wapiti (Immulyte, Diagnostic Products Corporation, Los Angeles, California; (McCorkell *et al.*, 2006). Intra-assay coefficients of variation were 9.7% for the low reference sample and 6.9% for the high reference sample. Serum concentrations of FSH were determined using a previously published and validated radioimmunoassay (McCorkell *et al.*, 2006, Rawlings *et al.*, 1984). Intra-assay coefficients of variation were 1.7% for the low reference sample and 7.8% for the high reference sample.

5.3.2 Experiment 2

Wapiti hinds (n = 13), 2 to 3 years of age and weighing between 168 to 214 kg, were used during the late anestrous period (August) and were maintained and handled as described in Experiment 1. On Day 0 (August 20), hinds were assigned randomly to 2 groups and were either given no treatment (control group, n = 6) or underwent follicle ablation (ablation group; n = 7). The procedure for follicle ablation was done using ultrasound-guided transvaginal follicle aspiration similar to that described for oocyte retrieval (Pieterse *et al.*, 1988, 1991) and follicle ablation (Bergfelt *et al.*, 1994) in cattle. Caudal epidural anesthesia was induced using 3 ml of 2% lidocaine and the perineal area was washed with surgical scrub before intravaginal insertion of a 7.5 MHz end-fire, convex-array transducer (Aloka SSD500). The transducer, equipped with a dorsally placed needle guide, was designed for transvaginal ultrasonography in women but was modified for transvaginal use in small ruminants, calves, camelids and cervids (Brogliatti *et al.*, 1996, 1997, 2000,) by incorporating modular extensions (Fig. 5.1). The transducer was positioned in the vaginal fornix by the operator and held with one hand while the free hand was used to transrectally position the ovary over the face of the transducer. A 19-guage, single lumen needle (55 cm long) was advanced through the needle guide until it made contact with the vaginal wall. The ovary was stabilized with the targeted follicle in the projected path of the needle, as displayed on the ultrasound monitor. The needle was then advanced through the vaginal wall

and into the antrum of the follicle. The follicular contents were aspirated and curettage was attempted by rotating the needle during aspiration. Follicular ablation was defined by the observed collapse of the targeted follicle during aspiration. The ovarian follicular response in both groups was monitored daily by transrectal ultrasonography, as described in Experiment 1, until Day 9 (August 29).

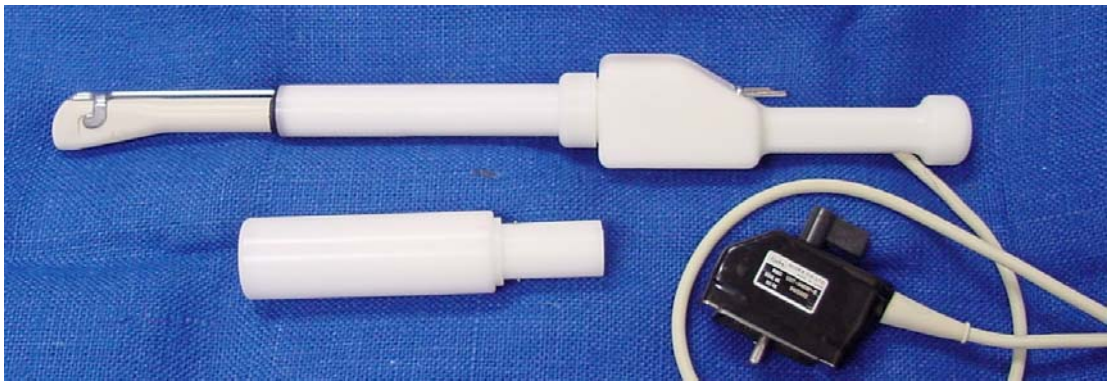

Figure 5.1. A 7.5 MHz convex-array ultrasound transducer with needle-guide originally designed for intravaginal use in women but custom-modified with modular extensions for intravaginal use in cervids.

5.3.3 Data analysis

A wave of follicle development was defined as the synchronous growth of a group of small follicles. The dominant follicle was defined as a follicle that attained a diameter ≥8 mm and exceeded the diameter of all others, and selection was defined as the day the dominant follicle became and remained larger than any of its cohorts. The day the dominant follicle of a wave was first detected at 4 mm in diameter was defined as the day of wave emergence.

Serial data were examined by analysis of variance for repeated measures using the PROC MIXED procedure of the SAS System (SAS System 8, Cary, NC) to determine the effects of day and group, and their interaction. Comparisons of non-serial data among groups were made by analysis of variance (Experiment 1) and the Student's *t*-test (Experiment 2). The degree of variability between groups was estimated by determining the absolute value of the difference between each data point and the group mean, and then comparing the groups by analysis of variance (Experiment 1) and the Student's *t*-test (Experiment 2). Comparisons between specific groups in Experiment 1 were made by least significant difference if a group effect ($P < 0.05$) was detected. All data are presented as the mean ± the standard error of the mean (SEM).

5.4 Results

5.4.1 Experiment 1

The effects of hormonal treatments on ovarian follicular wave emergence are shown in Fig. 5.2. The mean day of wave emergence did not differ between the control and estradiol alone groups, but tended to be later in the estradiol plus progesterone group (Day 4.0 ± 0.7, Day 3.5 ± 0.3, Day 5.2 ± 0.2, respectively; $P = 0.08$). Compared to the control group, the interval from treatment to wave emergence was less variable in the estradiol-17β alone group ($P = 0.07$) and the estradiol-17β plus progesterone group ($P < 0.05$).

Serum progesterone concentrations differed among the 3 groups (day-by-treatment interaction, $P < 0.05$; Fig. 5.3). Serum progesterone concentrations ranged from 0.3 ng/ml to 6.0 ng/ml even though no luteal tissue was identified during ultrasound examinations. In the groups that were not treated with progesterone, serum progesterone concentrations were not consistent within animal from day-to-day, and variance was greater ($P < 0.05$) than in the progesterone-treated group.

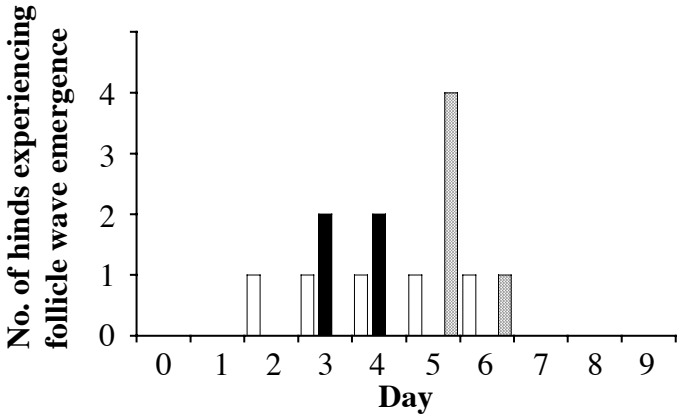

Figure 5.2. The day of ovarian follicular wave emergence in untreated wapiti hinds (open bars), and those treated with estradiol-17β (solid bars) or estradiol-17β plus progesterone (shaded bars).

Serum FSH concentration was influenced differently among groups (day-by-treatment interaction, $P < 0.05$; Fig. 5.3). FSH varied independently of day in the control group but a nonrandom pattern was observed in hormone-treated hinds ($P \leq 0.05$). Peaks ($P < 0.05$) in serum FSH concentrations were detected on different ($P < 0.05$) days in the hormone treatment groups; peaks were detected on Day 2 and Day 4, respectively, in the groups treated with estradiol-17β alone or estradiol-17β plus progesterone In both hormone treatment groups, FSH peaks preceded follicular wave emergence by 1 or 2 days.

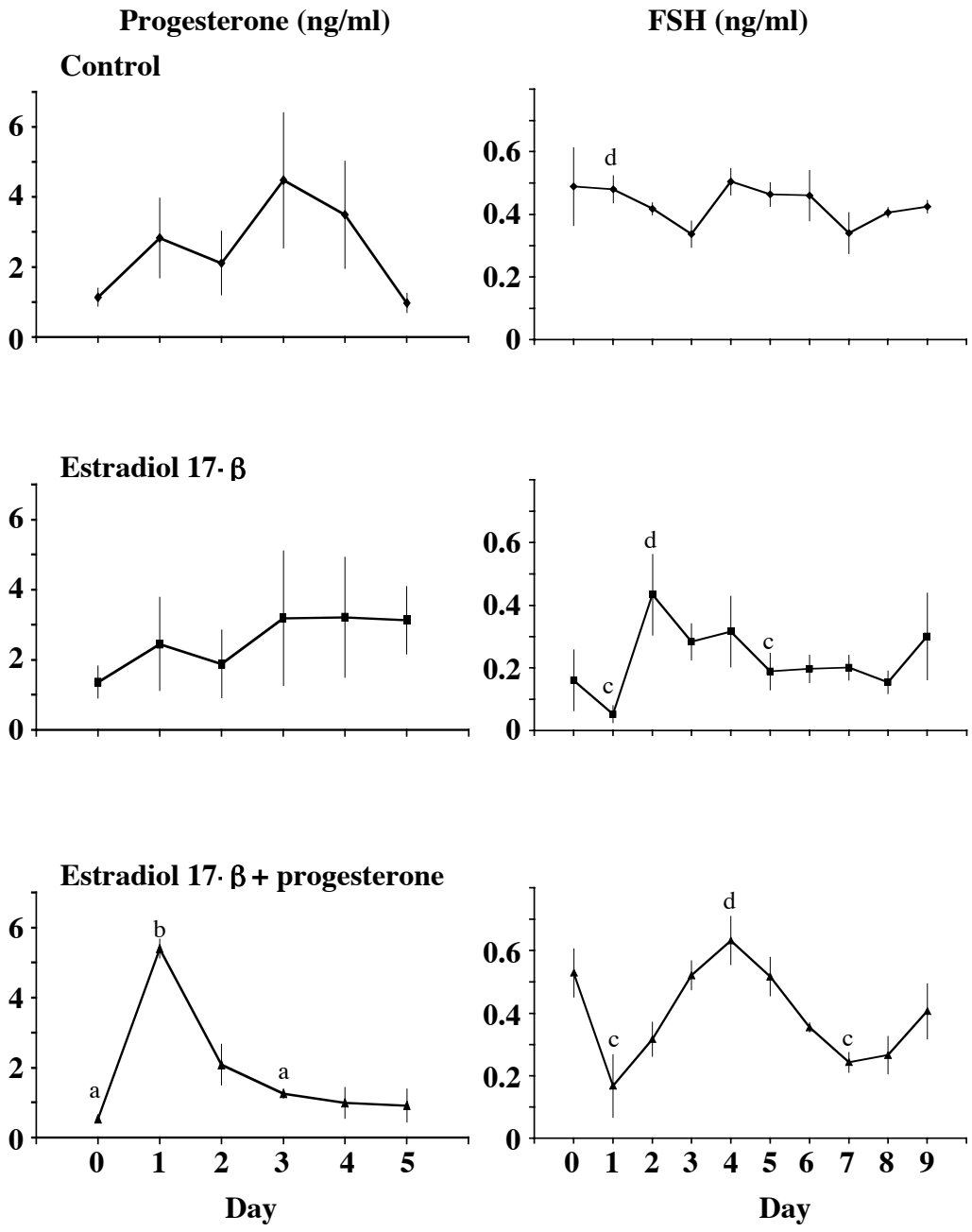

Figure 5.3. Daily serum progesterone and FSH concentrations for the 3 treatment groups: Group = controls, Group 2 = estradiol-17β, and Group 3 = estradiol-17β plus progesterone. Day 0 = day of treatment.
(a and b) among days, values with different superscripts are different ($P < 0.05$)

5.4.2 Experiment 2

The effect of follicle ablation on ovarian follicular wave emergence is shown in Fig. 5.4. The day of wave emergence was more variable ($P < 0.05$) and tended to be later ($P = 0.10$) in the control group compared to the ablation group (Day 2.5 ± 0.8 versus Day 1.4 ± 0.2).

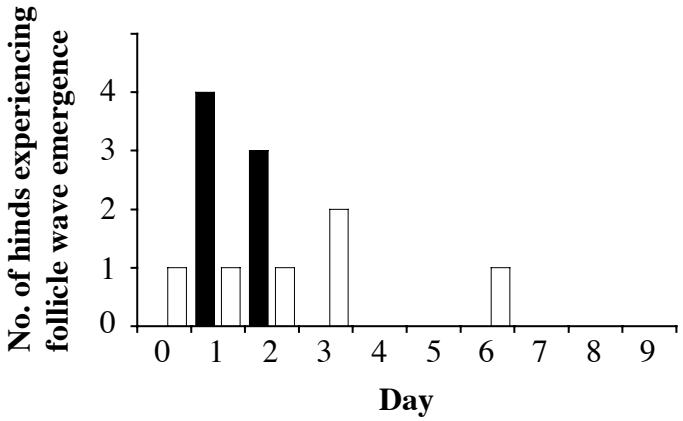

Figure 5.4. The day of ovarian follicular wave emergence in untreated wapiti hinds (open bars) and those that underwent transvaginal ultrasound-guided ovarian follicle ablation.

The follicle ablation procedure was well tolerated by the wapiti hinds. Follicles ≥ 5 mm in diameter were successfully ablated in all animals in the ablation group. The procedure was accomplished in less than 10 minutes per animal. In all but 1 hind, apparent hematomas at the ablation sites were detected during subsequent ultrasound examinations. Hematomas were not detected beyond Day 1 in 4 hinds, but were observed for 3 to 5 days in the 3 remaining hinds.

5.5 Discussion

During the estrous cycle in cattle, estradiol and progesterone work together to suppress LH release from the pituitary; estradiol reduced LH pulse amplitude and progesterone reduced LH pulse frequency (Price & Webb, 1988). When circulating concentrations of progesterone were low, treatment with estradiol alone was associated with a surge release of LH (reviewed in Bo *et al.*, 1995b). Hence, progesterone has been included in synchronization protocols in cattle to prevent an estradiol-induced release of LH, which has been shown to have adverse effects on follicular wave synchrony among animals (Bo *et al.*, 1994). In seasonal breeders, LH secretion is suppressed during the anestrous season by an enhanced negative feedback mechanism to estradiol (Anderson & Barrell, 1998, Karsch *et al.*, 1993). The inclusion of an estradiol-only group in the present study was based on the expectation that exogenous estradiol administered during the anestrous season in wapiti would not elicit release of LH, but instead, further suppress LH release from the pituitary gland.

The results of Experiment 1 support the hypothesis that treatment of wapiti hinds with estradiol-17β alone or in combination with progesterone will result in ovarian follicular wave emergence at a consistent time after treatment. The inclusion of progesterone, however, was associated with a significant delay in the FSH surge (2 days) and new wave emergence (2 days) in these anestrous-season hinds. In a previous study (McCorkell *et al.*, 2006), follicular wave emergence was detected ±1 day of the peak in serum FSH concentration. The peak in serum FSH in the estradiol plus progesterone group occurred 2 days

after the peak in the estradiol only group and the day of wave emergence was delayed correspondingly. Serum FSH concentrations reached nadir the day after estradiol treatment in both hormone-treated groups, and the concentrations increased on Day 2 in both groups. However the increase was slower and more sustained in the estradiol plus progesterone group, resulting in peak FSH concentrations on Day 4. The addition of progesterone modified the suppressive effect of estradiol, resulting in a slower and more sustained FSH response subsequent to treatment.

The mean serum progesterone concentration observed after treatment with 100 mg of progesterone im was 5.4 ± 0.3 ng/ml, which was higher than the maximum progesterone concentrations reported during the luteal phase of the estrous season (3.2 to 3.9 ng/ml) (McCorkell *et al.*, 2006). The observation that elevated serum progesterone concentrations, higher than those normally observed during the luteal period of the estrous cycle, may have an effect on serum FSH concentrations has been reported before (McCorkell, chapter 4). It is unclear, however, if the effects of progesterone on circulating FSH were direct, at the level of the pituitary, or indirect through suppression of the dominant follicle. In cattle treated with progesterone to induce below, normal, and above normal circulating luteal-phase concentrations of progesterone, it was concluded that progesterone influenced FSH secretion indirectly by suppressing the growing phase of the dominant follicle and abbreviating the period of follicular dominance (FSH suppression; Adams *et al.*, 1992).

Serum progesterone concentrations in untreated hinds in both experiments were elevated and some samples measured as high as 6.0 ng/ml, even though no luteal tissue was recognized during any ultrasound examinations. Elevations in progesterone were probably the result of stimulation of the adrenal gland due the stress of handling. Significant adrenal release of progesterone has been reported previously in white-tailed deer, fallow deer, and red deer (Asher *et al.*, 1989, Jopson *et al.*, 1990, Plotka *et al.*, 1983). The duration of elevated serum progesterone concentrations in these hinds is not known; however, it is expected that once the handling experience was completed the progesterone release would end and serum progesterone concentrations would decline as was found when concentrations were measured before and after anesthesia in white-tailed deer (Plotka *et al.*, 1983). The sharp elevation in serum progesterone concentration was expected after intramuscular injection, but it is noteworthy that although serum progesterone concentrations were elevated in all groups, the variance in serum progesterone was significantly lower in hinds treated with progesterone than in those that were not. This observation provides rationale for the hypothesis that adrenal progesterone secretion is restricted to brief episodes of stress.

Results of Experiment 2 support the hypothesis that physical ablation of ovarian follicles will elicit new wave emergence in wapiti, similar to the effect seen in other species (Bergfelt *et al.*, 1994, Ratto *et al.*, 2003). A new follicular wave emerged within 2 days of transvaginal ultrasound-guided aspiration of follicles ≥ 5 mm in all hinds in the present study, similar to the effect seen in cattle (Bergfelt *et al.*, 1994) and llamas (Ratto *et al.*, 2003). The only side-effect of follicle

ablation was the development of 1 or more post-aspiration hematomas within the antrum of aspirated follicles. Hematomas were detected as bright, very echogenic structures located at the site of the previously aspirated follicle. Only aspiration of follicles that were ≥7 mm in diameter resulted in hematomas. The hematomas were transient and did not appear to luteinize, although more detailed study is required to determine the extent to which aspirated follicles will retain or acquire hormonal function. The occurrence and morphology of post-aspiration follicular hematomas was similar to that reported previously in cattle in which the post-aspiration hematomas were found to be benign (Bergfelt *et al.*, 1994, Brown *et al.*, 1996).

It has been suggested that the variability in ovarian superstimulatory response in cattle is associated with the variability in follicular wave status at the time treatment is initiated (Armstrong, 1993, Nasser *et al.*, 1993). Superstimulatory treatments initiated in the presence of a functional dominant follicle (i.e., after selection) resulted in fewer ovulations (Adams *et al.*, 1993b, Huhtinen *et al.*, 1992, Pierson & Ginther, 1988). To date, attempts to induce ovarian superstimulation in wapiti have not to been very successful (Asher *et al.*, 1999, DeGrofft, 2000). Perhaps one reason for this is that previously unrecognized ovarian follicular wave status was not accounted for in the treatment design. Superstimulatory treatment initiated at the time of wave emergence, before selection of a dominant follicle, may result in an improvement in the ovarian response in wapiti.

Current estrus synchronization protocols for fixed-time AI in wapiti are empirical, based on protocols designed for sheep, and have resulted in pregnancy rates of ~70% (DeGrofft, 2000, Wenkoff, 2000). The calving rate following natural breeding in farmed wapiti was 93% (Friedel & Hudson, 1994), suggesting that the current protocol for fix-time insemination is not optimal. Critical examination of the effects of the empirically derived protocol on follicular wave dynamics (McCorkell, chapter 4) revealed that the progesterone treatment period was unnecessarily long, and that circulating progesterone concentrations were unnecessarily high. Improvement in such a protocol may be achieved by ensuring the presence of a new growing dominant follicle at the time ovulation is induced.

In conclusion, ultrasound-guided transvaginal follicle aspiration was found to be a viable method to ablate ovarian follicles ≥ 5 mm in wapiti hinds. This technique, as well as the administration of estradiol-17β alone or with progesterone resulted in the predictable emergence of a new wave of ovarian follicles 1.4 ± 0.4, 3.5 ± 0.3 and 5.2 ± 0.2 days later, respectively. All 3 treatments tested were successful in synchronizing ovarian follicular wave emergence among a group of anestrous wapiti hinds, and may be useful in estrus synchronization and superstimulatory protocols. An advantage of hormonal treatment is the lack of a need for technical expertise, and an advantage of follicular ablation is the rapidity of response.

5.6 References

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6.0 OVARIAN SUPERSTIMULATION FOLLOWING INDUCED FOLLICULAR WAVE EMERGENCE IN WAPITI (*Cervus elaphus*).

6.1 Abstract

The study was designed to evaluate 3 novel superstimulatory treatment protocols in seasonally anovulatory wapiti. Thirteen parous wapiti hinds, 2 to 4 years of age, were used late in the anestrous period (July). The ovaries were examined daily by transrectal ultrasonography. Hinds were treated on Day 0 with 5 mg of estradiol-17β im to induce a new wave of follicle development. At the expected day of wave emergence (Day 3), hinds were assigned randomly to 3 treatment groups and given 100 mg FSH im once a day for 4 days (n = 5), 200 mg FSH subcutaneously on Day 3 and Day 5 (n = 4), or 200 mg FSH plus 2.5 mg LH subcutaneously on Day 3 and Day 5 (n = 4). All hinds were given 10 mg LH im on Day 6 to induce ovulation. Results did not support the hypothesis that less frequent treatment (i.e., reduced stress of handling) and supplementation with LH improved the superovulatory response. The mean number of ovulations per animal in the respective groups was 6.2 ± 2.0, 15.5 ± 5.9, and 14.8 ± 2.7, and was not different among groups. In conclusion, the technique of inducing a follicular wave and initiating superstimulatory treatment at the time of wave emergence was effective in wapiti. The most efficient method of ovarian superstimulation in this study involved the administration of estradiol-

17β on Day 0 followed by 200 mg FSH sc on Days 3 and 5, and induction of ovulation on the evening of Day 6 with 10 mg of LH. Compared to conventional methods that require 14 days and handling the hinds 6 times, the protocol used herein reduced the treatment period to 8 days and the number of animal handlings to 4.

Introduction

Interest in the use of assisted reproductive technologies in wapiti has increased with the rising importance of cervid species in agriculture. The desire to propagate genetically valuable animals in farmed or game ranch populations has led to the adoption of artificial insemination and embryo transfer. Artificial insemination has been applied successfully in red deer and wapiti (Asher *et al.*, 1993, 2000, Wenkoff, 2000), but the results of attempts at embryo transfer appear to be more species specific. In contrast to the relative success of embryo transfer in red deer, in which 3 pregnancies per donor have been reported (Fennessy *et al.*, 1994), the application of embryo transfer has been less successful in wapiti (DeGrofft, 2000, Wenkoff & Bringans, 1991).

Conventional methods of ovarian superstimulation in the conspecific red deer include the placement of a progesterone-releasing device (CIDR) intravaginally for 12 days to synchronize estrous cycles (Fennessy *et al.*, 1994). Superstimulatory doses of FSH are then given twice-daily beginning on the eighth day after CIDR placement and ending 12 to 24 hours after CIDR removal (Fennessy *et al.*, 1994). The period of CIDR placement is intended to be sufficiently long to ensure regression of any existing CL prior to CIDR removal. A common addition to the treatment protocol is eCG, which is intended to reduce the variability in response (Asher *et al.*, 1995, Fennessy *et al.*, 1989, Scott *et al.*, 2000). Equine chorionic gonadotropin is commonly given with the last FSH treatment but others reported improved results when it was given with the first treatment of FSH (Berg *et al.*, 1995).

The status of the ovarian follicular wave at the time superstimulatory treatment is initiated has been found to influence the superstimulatory response in cattle (Adams, 1994, Adams *et al.*, 1993, 1994, Huhtinen *et al.*, 1992, Nasser *et al.*, 1993) and sheep (Rubianes *et al.*, 1997). The ovarian response when treatment was initiated during the period of follicular dominance was reduced compared to treatment initiated at the time of follicular wave emergence. Initiating treatment as little as one day after wave emergence significantly reduced the superstimulatory response compared to treatment initiated the day before or the day of wave emergence (Adams *et al.*, 1994, Nasser *et al.*, 1993). The influence of follicular wave status at the time of ovarian superstimulation has not been evaluated in cervid species.

In a recent study in wapiti, 85% of the estrous cycles were composed of 2 or 3 follicular waves, the remainder had 4 waves, and the duration of the cycle was positively correlated with the number of waves (McCorkell *et al.*, 2006). Based on follicular dynamics in wapiti, and the effects of follicular dominance in other species, we estimate that there are only 4 to 6 days during the estrous cycle when superstimulatory treatments may be initiated for optimal results. Given that the estrous cycle in 2-wave hinds is 20 days (McCorkell *et al.*, 2006), initiating superstimulatory treatment without regard to follicular wave status would result in suboptimal results 80% of the time.

Ovarian follicular wave emergence has been induced in cattle by the removal of follicular dominance using hormonal treatment (estrogen and progesterone; Bo

et al., 1993, 1994, 1995a, 2000, Caccia & Bo, 1998, Mapletoft *et al.*, 1999) or physical ablation by transvaginal ultrasound-guided follicle aspiration (Bergfelt *et al.*, 1994). Both of these methods have been used to improve the response to superstimulatory treatment in cattle (Baracaldo *et al.*, 2000, Bergfelt *et al.*, 1997, Bo *et al.*, 1995b). The results of one study done in red deer (Scott *et al.*, 2000) lend support to the hypothesis that the same relationship between follicular dominance and superstimulatory response exists in cervids. Superstimulatory treatment initiated on the day after expected ovulation (ovulation coincides with wave emergence; Ginther *et al.*, 1989, McCorkell *et al.*, 2006) in red deer hinds that were treated to synchronize ovulation, resulted in higher ovulation rates than in hinds treated in the conventional manner. In this regard, authors of a recent study in wapiti reported on the effectiveness of a method to electively induce follicular wave emergence directly, without the necessity of inducing ovulation (McCorkell, chapter 5).

Two other factors have been identified as potential suppressors of the ovarian superstimulatory response in wapiti. The first is the amount of animal handling that is required to administer the treatment protocols. A poor superstimulatory response was attributed to the stress of animal handling in one study done in wapiti (Wenkoff & Bringans, 1991). In red deer, fallow deer, and white-tailed deer, the adrenal gland has been identified as an important source of progesterone (Asher *et al.*, 1989, Jopson *et al.*, 1990, Plotka *et al.*, 1983), and release of progesterone has been associated with animal handling (Jopson *et al.*, 1990). Progesterone has been shown to have a negative effect on follicle development; progesterone suppressed the growing phase of large follicles in a

dose-dependent manner (Adams *et al.*, 1992). The second factor is the presence of LH in the FSH preparation used for superstimulatory treatments. In one report, better results were obtained when wapiti were superstimulated with an FSH preparation that also contained LH rather than the more purified FSH preparations with low LH activity (Wenkoff & Bringans, 1991). Empirically, eCG has been incorporated into conventional superstimulation protocols in cervids because of its FSH- and LH-like activity.

The present study was designed to evaluate 3 novel ovarian superstimulatory treatment protocols in wapiti, and to test the hypothesis that the response to treatment is inversely proportional to the frequency of treatment (animal handling) and the purity of FSH (lack of LH) treatment.

6.3 Material and methods

Thirteen parous wapiti hinds, 2 to 4 years of age, were used during July (late anestrous period). The hinds were maintained on alfalfa pasture in a 4-hectare pen on a farm near Saskatoon, Saskatchewan (52°07′N, 106°38′W). For examination, the hinds were moved from the pen through a 300 meter-long alley to the handling facility. The hinds were restrained in a squeeze chute for examination and treatment. The ovaries were imaged by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD500, Instrument for Science and Medicine, Vancouver, Canada). During examination, drawings of each ovary were made to record the size and location of all follicles ≥ 4 mm in diameter (Knopf *et al.*, 1989, McCorkell *et al.*, 2004).

Hinds were treated with 5 mg of estradiol-17β, dissolved in a volume of 1 ml of canola oil (Bo *et al.*, 1995b), im (Day 0) to induce a new wave of follicle development. On Day 3, the expected day of wave emergence (McCorkell, chapter 5), the hinds were assigned randomly to 3 superstimulatory treatment groups and given 1) 100 mg FSH (Folltropin-V, 20mg/ml NIH-FSH-P1, Bioniche Animal Health Inc. Belleville, Ontario, Canada) intramuscularly once a day, in the morning, for 4 days (FSH daily; n = 5), 2) 200 mg FSH subcutaneously (caudal to the midpoint of the left scapula) on the morning of Day 3 and Day 5 (FSH D3&5; n = 4), or 3) 200 mg FSH plus 2.5 mg LH (Lutropin®-V, 5 mg/ml Armour standard of LH, Bioniche Animal Health Inc. Belleville, Ontario, Canada) subcutaneously (caudal to the midpoint of the left scapula) on the morning of Day 3 and Day 5 (FSH/LH D3&5; n = 4). All hinds were given 10 mg of LH intramuscularly on the evening of Day 6 to induce ovulation. Ultrasonographic examinations of the ovaries were done daily from Days 0 to 3 and from Days 6 to 8. Ovulation was defined as having occurred when a follicle > 6 mm identified the previous day could no longer be identified at subsequent examinations.

6.3.1 Data analysis

Data are presented as the mean ± the standard error of the mean (SEM). Comparisons between groups were made using ANOVA.

6.4 Results

Ovarian follicular superstimulation was evident in all hinds in the study, but one hind in the FSH D3&5 group failed to ovulate by Day 8. The number of follicles ≥6 mm and the maximum follicle diameter on Day 6 was similar among groups (Table 6.1). No differences among groups were detected in the number of ovulations, interval to ovulation, or the number of unovulated follicles on Day 8 (Table 6.1).

Table 6.1. Ovarian response to three different superstimulatory treatment regimens in wapiti.

Treatment group*	n	Number of follicles ≥6 mm on Day 6 (Range)	Maximum follicle diameter (mm)	Number of ovulations (Range)	Interval to ovulation	Number of follicles ≥6 mm on Day 8 (Range)
1) FSH im once a day	5	12.8 ± 2.7 (7 – 23)	9.0 ± 1.1	6.2 ± 2.0 (3 – 14)	8	6.6 ± 1.4 (2 – 10)
2) FSH sc on Days 3 and 5	4	18.5 ± 3.6 (11 – 26)	8.5 ± 0.3	15.5 ± 5.9 (0 – 26)	8	3.0 ± 2.7 (0 – 11)
3) FSH+LH sc on Days 3 and 5	4	17.8 ± 1.7 (15 – 22)	10.8 ± 1.5	14.8 ±2.7 (8 – 21)	8	3.0 ±1.6 (0 – 7)

*For each group, the total dose of FSH (Folltropin) was 400 mg. No differences were detected among groups for any end point.

6.5 Discussion

The study was conducted in the late anestrous period of the annual reproductive cycle. This time period is most advantageous because calves can be weaned from the hinds that are going to be treated, thereby reducing animal handling problems. In addition, the breeding season is very busy if artificial insemination and embryo transfer is contemplated and therefore producing embryos prior to the onset of the breeding season is more efficient. Finally, the embryos that are produced will be available for transfer during the breeding season and the donor hinds will still have an opportunity to become pregnant.

The results of this study did not support the hypothesis that the response to treatment is inversely proportional to the frequency of treatment (animal handling) and the purity of FSH (lack of LH) treatment. There was no indication that a reduced frequency of treatment or the addition of LH was beneficial to the ovarian response. However, all three protocols of ovarian superstimulation were effective and results were comparable to those reported in red deer. The mean number of follicles ≥6 mm at the end of treatment and the mean number of ovulations for all groups combined in the present study were 16 .0 ± 1.7 and 12.7 ± 2.2, respectively, which compares favorably with results of previous studies in red deer using conventional methods where the number of follicles and the number of ovulations ranged from 10 to 15 and 5 to 11, respectively (Asher *et al.*, 1995, 1997, Berg *et al.*, 1995). Conventional methods of superstimulation in wapiti have resulted in low embryo production compared to red deer (Bringans, 1989, DeGrofft, 2000, Wenkoff & Bringans, 1991), but the

reason for lower embryo production in wapiti is not clear. Whether or not it is due to a poor ovarian response to superstimulatory treatment is unknown because previous studies were not designed to record follicle numbers or ovulations subsequent to treatment.

While the number of hinds in this study was limited and a study on a larger group may reveal differences in the future, based on this study the best method of superstimulating follicle development in wapiti was that used on Group 2 hinds. The addition of LH to the superstimulatory treatment produced no more follicles nor did it increase the ovulation rate and therefore is of no advantage. The subcutaneous route for FSH administration resulted in a similar ovarian follicle response to the intramuscular route, but required 2 fewer injections. Therefore, to minimize animal handling and treatment costs, the most efficient method in this study involved the administration of estradiol-17β on Day 0 followed by 200 mg FSH sc on Day 3 and a further 200 mg of FSH sc on Day 5 and 10 mg LH on the evening of Day 6 to induce ovulation.

This study was conducted during the anestrous season when the negative feedback of estradiol on pituitary gonadotropin release is enhanced, thereby making a pre-ovulatory release of LH unlikely (Anderson & Barrell, 1998, Meikle & Fisher, 1996). If this method was used in the estrous season, it is likely that precautions would need to be taken to prevent premature release of LH from the pituitary in response to elevated concentrations of estradiol from the large number of ovarian follicles developing within the ovaries. In addition, during the estrous season the presence of a CL also needs to be considered. The

release of LH could be controlled by using CIDR devices containing progesterone, which would be inserted on Day 0 at the time of estradiol treatment and removed at the time of LH treatment on Day 6. To ensure that a functioning CL was not present at the time of CIDR removal it would be necessary to incorporate a treatment with prostaglandin on Day 5 at the time of the second FSH treatment. Using this modified protocol, it should be possible to superstimulate wapiti over a period of 8 days while only handling the animals 4 times. This compares to the present conventional method that requires 14 days and handling the hinds 6 times. Similar superstimulatory protocols are presently being used successfully in cattle (Bo *et al.*, 2002).

The use of daily ultrasound examinations may have confounded the effect that reduced superstimulatory treatments had on the results of this study. Removing the ultrasound examinations would allow the effect of increased handling required by the daily FSH injections given to Group 1 hinds to be more clearly revealed. Not all hinds reacted in the same fashion to being handled. One hind in Group 2 failed to ovulate any follicles. This hind displayed evidence of stress when being handled and would lay down in the squeeze chute prior to every examination. It is possible that her reaction to being handled resulted in increased progesterone release as has been reported in red deer (Jopson *et al.*, 1990). This endogenous release of progesterone may have reduced the rate of follicle development so that the follicles were not mature enough to respond to an LH surge and ovulate. If this animal's results are excluded the number of non-ovulated follicles seen in Group 1 hinds are greater than ($P \leq 0.05$) the number of non-ovulated follicles observed in the other 2 groups and the

number of ovulations observed are fewer ($P \leq 0.05$). This finding supports the hypothesis that a reduced number of superstimulatory treatments would result in increased ovarian follicle development. Additionally it could be interpreted to indicate that the s.c. route of FSH administration was superior to the i.m. route given the confounding effect of daily ultrasound examinations. These results provide impetus for a more critical comparison of the superstimulatory response relative to dose frequency and route using a greater number of animals.

In conclusion, the technique of inducing a follicular wave and initiating superstimulatory treatment on or before the follicular wave was expected to emerge was effective in wapiti. This technique has the advantage of reducing the treatment period by 6 days and the number of times the animals are handled by one third over the conventional method. The most efficient method of ovarian follicle superstimulation in this study involved the administration of estradiol-17β on Day 0 followed by 200 mg FSH subcutaneously on Day 3 and 5, and the induction of ovulation on the evening of Day 6 with 10 mg of LH.

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7.0 General Discussion

Several objectives were set out in the introduction; the first general objective was to increase our understanding of the endogenous control of ovarian function in wapiti. To satisfy that objective ovarian follicle and luteal function were studied during the estrous season and the periods of seasonal transition. During the estrous season follicle development was found to occur in a wave-like manner and 85% of the animals studied had 2 or 3 follicular waves per IOI, the remainder had 4 waves. On all occasions follicle waves were preceded by a surge in serum FSH concentration that peaked on the day follicle wave emergence was detected. Selection of the dominant follicle was evident the day after follicular wave emergence and happened at about the same time as the first drop in FSH concentration. In the majority of hinds (hinds with 2- or 3-wave IOI), the dominant follicles of the first follicular wave after ovulation were very similar and the first interwave intervals were the same length. The CL were also found to have similar characteristics, including the day on which they began to regress. However, the length of the interovulatory interval depended on the number of follicular waves and was longer in 3-wave hinds.

Follicular and luteal dynamics during the seasonal transitions were found to be most different from the breeding and nonbreeding seasons during the fall transition into the breeding season. In most hinds the first ovulation of the

breeding season was followed by an IOI that was about half as long as subsequent IOI. During the first IOI, only 1 wave of follicle development occurred and there was only a small CL that produced low serum concentrations of progesterone for only a few days. Serum FSH concentrations remained temporally related to dominant follicle emergence. It was only at the time of the first or second ovulations of the year that multiple ovulation was observed; 20% of the hinds had two or more ovulatory follicles. The follicle and luteal dynamics of the winter transition out of the breeding season differed very little from those observed during the breeding season. The last IOI of the annual reproductive cycle was found to have 2 or 3 follicular waves and was of the same duration as previous IOI. One characteristic that may reflect a seasonal change was the maximum diameters of the last 2 ovulatory follicles, which were smaller than the maximum diameters of the first 2 ovulatory follicles during the fall transition. The CL from the last ovulation of the breeding season was detected for a longer period but the day it began to regress was only 2 days later than the CL from the previous IOI. There was no evidence of luteinization of unovulated follicles, nor was recrudescence of ovulation detected in the period following the last ovulation.

This new information about follicle and luteal dynamics when combined with information about the anovulatory season (McCorkell *et al.*, 2004) provides a more complete understanding of ovarian function in wapiti. Ovarian follicle development follows a regular pattern throughout the annual cycle. Repeated follicle waves develop in response to periodic stimulation by surges in serum FSH concentration. One area that is still not clear is whether or not individual

animals have a specific periodicity unique to them (i.e. one animal has repeated follicular waves every 9 days whereas another animal may consistently have follicular wave intervals of 7 days).

A description of the annual pattern of follicle and luteal dynamics is now possible for the non-pregnant hind. Beginning during the middle of the anovulatory season (June in North America) ovarian follicle waves occur, on average, every 6 days and the maximum diameter of the dominant follicle at that time is 7 mm (McCorkell *et al.*, 2004). This is the time period when the days are the longest and LH levels are expected to be very low due to seasonal cues that are maintaining the suppression of GnRH pulses.

Toward the end of the anovulatory season and approaching the first ovulation of the breeding season, regular follicular waves persist but there is evidence that the interval between follicular waves increases to 8 days and the maximum diameter of the dominant follicle increases to 11 mm (McCorkell *et al.*, 2004). This change may be due to increased serum concentrations of LH that reflect the relaxation of the suppression of GnRH pulses due to the effect of the circannual rhythm of reproduction that was initiated by the long days of late spring, and becomes evident at the onset of the breeding season.

The increased concentrations of LH will permit the dominant follicle to grow for a longer period of time and hence reach a larger diameter (Ginther *et al.*, 2000). The larger diameter of the dominant follicle is associated with a great increase in the number of granulosa cells lining the follicle. The granulosa cells respond

to the increased concentrations of LH by producing estradiol (Ireland & Roche, 1983). At some point, which occurs near the autumnal equinox in the animals studied, the combination of reduced suppression of the GnRH pulses and the increased size of the dominant follicle stimulate the production of sufficient estradiol to trigger a surge in LH that precipitates the first ovulation of the breeding season.

This ovulation causes the emergence of a new follicular wave and the formation of a transient, small and hypo-functional CL in most animals. The CL only produces a low concentration of progesterone for a few days. The dominant follicle of that follicular wave ovulates shortly after the CL regresses. The first IOI, consists of 1 follicular wave and is similar in duration to the interwave interval of the late anovulatory period.

The second ovulation of the breeding season is followed by subsequent ovulations that are separated by 2 or 3 follicular waves and the development and regression of a single CL. The estrous cycle will be repeated approximately 7 times before the onset of the anovulatory season. The only difference between the first and last IOI is a slight reduction in the maximum diameter of the ovulatory follicle towards the end of the breeding season. The reduction in follicle diameter may be due to declining serum concentrations of LH. Near the end of the breeding season at the time of the winter solstice, the increasing serum concentration and duration of melatonin, due to short days, is stimulating the process that leads to an enhanced negative feedback of estradiol on the hypothalamo-pituitary axis. This process restricts the number of GnRH

pulses resulting in declining release of LH from the pituitary. At some point, usually before the spring equinox, the suppression of LH release is sufficient to block the LH surge needed to cause ovulation and the anestrous season begins.

When ovulation fails, regular follicular waves continue every 7 days with the dominant follicles attaining a maximum diameter of 10 mm. During the interval from the beginning of the anovulatory season to the summer solstice, it appears that the maximum diameter of the dominant follicle continues to decline and the interwave interval shortens by 1 day, which may indicate a continued suppression in LH release.

The second general objective was to explore exogenous control of ovarian function with regards to the augmented understanding of endogenous control. The current treatment used to synchronize estrous in deer in general, and wapiti in particular, was studied to discover its effect on ovarian function. It was found to successfully synchronize estrus but that it accomplished this at the expense of ovarian resources and time. The first follicular wave that developed after treatment regressed and a second follicular wave occurred before the protocol allowed ovulation to take place. However, the success of the protocol still depended on manipulation of the pattern of regularly repeating follicular waves. The protocol synchronized follicular wave emergence in the treated group of animals thereby facilitating synchronous ovulation.

Periodic follicular waves are a fundamental factor in ovarian function and treatment protocols designed to manipulate them are powerful tools. Two

strategies that have been used in cattle to control follicular wave emergence, hormonal treatment and physical ablation, were examined in wapiti. They were equally effective in wapiti as in cattle. The emergence of a new follicular wave was found to occur at a predictable time after treating anestrous wapiti hinds either hormonally with estradiol-17β or estradiol-17β plus progesterone or by ultrasound guided transvaginal follicle ablation.

The application of one these tools was examined in a study to evaluate three novel ovarian superstimulatory treatment protocols in wapiti. Previous research had shown that wapiti did not respond to conventional superstimulation protocols used in red deer (Asher *et al.*, 1999, DeGrofft, 2000). Hormonal treatment (estradiol-17β) was used to control wave emergence and the follicular wave was stimulated by the use of FSH in three different ways. All treatment groups responded similarly and were successfully superstimulated. The response of the wapiti hinds to the superstimulatory protocols in this study was equivalent to superstimulatory response reported previously in red deer.

The principal hypothesis that guided the preceding studies was that it was necessary to characterize ovarian function during the four seasons of the annual reproductive cycle and from this knowledge novel approaches of exogenous control of ovarian function might emerge. Ovarian function has been characterized in the four seasons, three seasons of which were completed in the course of the studies presented here. The follicle and luteal dynamics are now known in this species for all seasons of the year. Furthermore this knowledge

will provide a template upon which other species of deer can be compared. It is likely that closely related species will have very similar ovarian follicle and luteal dynamics and this information may be directly applicable. In other more distantly related species, the ovarian follicle and luteal dynamics may not be as similar but they will likely have some commonalities that will be useful in understanding their reproductive physiology. The detailed understanding of ovarian follicle and luteal dynamics reported in these studies will be a resource not only for further studies in wapiti but for all species of deer.

The results of the third study illustrate that more study is often necessary to successfully deal with a problem. The objective of the protocol examined was to synchronize estrus. However, the desired goal was to synchronize ovulation. It turns out that the protocol was largely successful but the previous explanation for its success was incorrect. There is no ever-ready pool of ovarian follicles from which one follicle may be selected based on its maturity at the onset of luteolysis. Application of this protocol has led to poor results in the superstimulation of wapiti and to the acceptance of a method for estrous synchronization that may not be optimal. Now that ovarian follicle development is better understood, new treatment protocols can be developed that promise to be more efficient and effective.

The final two studies report on successful and novel methods of ovarian follicle manipulation in wapiti that are based on knowledge gained in the earlier studies. The ability to control ovarian follicle development increases the potential for success when applying reproductive technologies by ensuring the

oocytes that are either ovulated or collected are at an optimal stage of development for the procedure being contemplated. Furthermore, success in wapiti should lead to the successful application of these methods in other species of deer. This has great implications for both farmed and wild species. In agriculture the exchange of gametes and embryos is more convenient and less costly than actually transferring livestock and provides far less risk of disease transfer (Stringfellow & Givens, 2000). For the conservation of threatened species, both captive and wild, the collection of gametes or embryos provides a method for maintaining genetic diversity and the insurance of banking germplasm in case of a catastrophic event.

Several observations recorded throughout the course of the studies presented were unexpected and warrant further investigation. One of the most interesting occurred during the transition from anestrous to estrous seasons when multiple ovulations were observed and resulted in the development of multiple CL within the ovary. Multiple ovulations were not observed at any other time during the annual reproductive cycle. Multiple CL have been reported before in studies of ovaries collected from pregnant hinds. In these studies the smaller CL was referred to as an accessory CL (Morrison, 1960). The multiple ovulations observed at the beginning of the breeding period offer one possible explanation to the origin of the accessory CL. If a CL that is functionally similar to the CL of the long IOI of the breeding season follows multiple ovulations, multiple embryos could develop. Twin pregnancies are reportedly rare (Friedel & Hudson, 1994, Guinness *et al.*, 1971, Guinness *et al.*, 1978) yet the occurrence of accessory CL has been reported as high as 60% (Morrison, 1960). Further study

is needed to determine if multiple ovulations lead to the development of multiple embryos and then in turn if embryo reduction occurs to result in a predominance of single calves.

Multiple ovulations may indicate a change in the process of dominant follicle selection. During the breeding season a single dominant follicle was selected from a follicle wave and only single follicles ovulated demonstrating the effectiveness of the selection process and its ability to control the number of follicles allowed to develop. However during the fall transition into the breeding season this process does not appear to be operating as effectively as in some cases more than one follicle is allowed to develop and ultimately ovulate. It is possible during this time period that the process of selection is undergoing some adjustment as well. Selection is manifest through the presumptive dominant follicle's development of LH receptors and its suppression of FSH concentrations by the production of estradiol and inhibin (Adams *et al.*, 1993, Ginther *et al.*, 2001). The development of LH receptors allows the follicle to continue its development in an environment with low FSH concentrations and to continue to suppress FSH secretion even further, starving other developing follicles that have not yet developed LH receptors, which eventually results in their demise (Ginther *et al.*, 2001). Further study is needed to determine what allows the development of multiple ovulatory follicles during the fall transition and whether or not this involves the serum concentrations of FSH and LH and or the development of LH receptors by the developing follicles.

In most cases, a short IOI and small hypo-functional CL followed the first ovulation of the breeding season. This observation has also been made in cattle following parturition and the resumption of ovulation. In cattle, the exposure to elevated progesterone concentrations appears to be necessary for the development of a subsequent luteal phase of normal duration and the estrous behaviour (Adams, 1999). Multiple ovulations in wapiti occurring at the time of the first ovulation may be part of a mechanism to ensure that elevated progesterone concentrations ensue ("progesterone priming") and therefore precipitate strong estrous behaviour and the production of a luteal phase that will sustain pregnancy.

Estrus was recognized as an important endpoint in the study during the fall transition and the resumption of ovulation. However, estrus detection in wapiti and deer in general is difficult (Guinness *et al.*, 1971, Morrison *et al.*, 1959) because behavioural displays of estrus are of short duration and discreet. An attempt was made to record estrous behaviour using a commercially available automated system designed for cattle called HeatWatch® (Cowchips, LLC Denver, Colorado). The HeatWatch® system uses a transmitter that is attached to the tail-head of each female and is activated by the male when he mounts and depresses a small button on the transmitter with his sternum. Each transmitter sends a unique signal to a receiver where the information is stored until it is downloaded to a computer for analysis. When functioning properly the system will record the date, time, duration and identity of the female for each mount by the male. During the first fall in which transition data were recorded,

transmitters were attached to all of the hinds and a vasectomized stag was placed in the pen. However, no mounts were recorded even though mounts were observed to occur. Data collection was abandoned. The problem appears to have been related to the positioning of the transmitter on the female and the size of the button needed for the stag to depress in order to send a signal. Modifications to the transmitters were made prior to the second session of data collection the subsequent fall. Unfortunately, data collection by the system was still unreliable and useful information was not collected. Further modification to the transmitters has been made and successful data collection is now possible. Future studies should be able to include information about estrous behaviour in relation to follicle development and ovulation.

The ultimate goal of superovulation is to produce embryos and this important endpoint was to be included in the study on ovarian superstimulation in wapiti. However, non-surgical collection of embryos in wapiti proved to be very difficult. The cervix in wapiti is long and of small diameter in the non-estrous female. The many circular folds of the cervix further increase the difficulty of passing a pipette through the cervix into the uterus. The technique was attempted on several individuals with poor results and was therefore not included in the study. Surgical collection of embryos either by laparoscopic or midline laparotomy has been reported (Argo *et al.*, 1994), but the use of these techniques was not possible at the time the study was conducted. Future studies will need to incorporate some form of reliable embryo collection or IVF in order to evaluate the fertility of the oocytes that are produced from the superstimulation techniques that the study showed were successful.

Manipulation of follicle development and superovulation was conducted in the late anestrous season. This time period was chosen for several reasons. The first was that hinds with calves at foot could be weaned allowing for their participation in the study without the concerns of injuring the calf because of the extra handling required. Secondly, this time period offers the possibility of producing oocytes and embryos that would then be available for the upcoming breeding season and would permit the hinds in the study to rejoin the breeding herd and become pregnant. It was also felt that producers would favor this time period rather than during the breeding season that is already very busy, especially if other procedures like AI are being conducted. The results of studies conducted at this time of year have limitations in their ability to reflect the result that might be expected during the breeding season. The reproductive system is still under the inhibitory effects of seasonal regulation, some of which are known like the reduced pulse frequency of LH but other factors which may effect oocyte competence and therefore subsequent embryo production are less well understood. The effect of season has been noted in red deer; oocytes collected late in the breeding season and subjected to IVF failed to develop to the blastocyst stage and a decreasing percentage showed evidence of cleavage as the breeding season progressed (Berg *et al.*, 2003).

It is known that the anestrous period is characterized by regular anovulatory follicular waves (McCorkell *et al.*, 2004), however some reports suggest that the factors that inhibit ovulation are not acting uniformly over the whole time period (Curlewis *et al.*, 1991, Meikle & Fisher, 1996). The pattern described by the factors inhibiting ovulation may have an effect on the viability of embryos

derived from oocytes collected during the anestrous period and is therefore important to understand. Exactly what the pattern of ovulation suppression is during the anestrous season is unknown but many studies indicate that ovulation is most strongly suppressed early in the anestrous period (Brinklow *et al.*, 1992, Curlewis *et al.*, 1991, McLeod *et al.*, 1991, Meikle & Fisher, 1996), perhaps to ensure that ovulation does in fact cease. For example, ovulation in response to exogenous GnRH is less likely during the early anestrous period in Pere David's Deer (Brinklow *et al.*, 1992, Curlewis *et al.*, 1991, McLeod *et al.*, 1991) and red deer (Meikle & Fisher, 1996), and the response to superovulatory regimens is reduced in the early anestrous period in red deer (Asher *et al.*, 2000b).

Some information exists that indicates that the effect on ovulation rate and oocyte competence may be minimal in the late anestrous period and supports the use of this time period for the collection of oocytes and embryos. In one study the percentage of oocytes collected from wapiti hinds immediately prior to the breeding season that were suitable for IVM was similar to another study which used repeated oocyte collection in red deer (64% for wapiti vs 58% for red deer (Berg *et al.*, 2003). Further, the cleavage rate attained in the oocytes collected from wapiti and subjected to IVM was 38% compared to the cleavage rate of 41% in red deer in the same studies. Finally the percentage of oocytes collected that went on to develop into blastocysts was 16% in the study using wapiti and ranged from 13 to 18% in red deer (Berg *et al.*, 2003). In another study using multiple ovulation and embryo transfer (MOET) protocols on red deer hinds (Asher *et al.*, 2000b), the highest ovulation rate occurred in the 4

weeks preceding the normal date of the first ovulation (Asher *et al.*, 2000a) of the breeding season and the ovulation rate in the month before that was similar to results found during the middle of the breeding season. Further research is needed to determine whether or not the late anestrous season is suitable for embryo production and to determine what, if any, are differences in the ovarian response to manipulation of follicle development.

A comparison of ovarian follicle and luteal dynamics in wapiti with those reported for cattle, sheep and goats suggests that during the ovulatory season wapiti more closely resemble cattle than either sheep or goats. Cattle, like wapiti, typically have 2 or 3 follicular waves during one IOI that is 20 and 23 days in length, respectively (Ginther *et al.*, 1989a, Ginther *et al.*, 1989b). Sheep and goats usually have 4 waves of follicle development during one IOI, which is 16 to 17 days long in sheep (Ravindra *et al.*, 1994) and 23 days long in goats (Ginther & Kot, 1994). In cattle, as in wapiti, the dominant follicle is clearly identifiable; however, in sheep and goats follicular dominance is difficult to recognize for follicular waves occurring in the middle of the IOI. It has been suggested that follicular waves in sheep and goats are better characterized as major or minor waves (Adams, 1999). The maximum diameters of the dominant follicle in cattle are larger than in wapiti and smaller in sheep and goats (16, 7, and 10 mm, respectively).

Sheep are seasonally polyestrous and have been studied during the seasonal transitions. In two reports where ovarian function has been monitored by ultrasonography, the transition into the breeding season from the anestrous

season was characterized by a small transient increase in progesterone concentration that was not correlated with ovulation or a luteal structure in the ovary (Bartlewski *et al.*, 1999, Ravindra & Rawlings, 1997). In wapiti, the transient increase in progesterone was always preceded by an ovulation, which was followed by the development of a small CL. Although cattle are not seasonal breeders, the interval between parturition and the resumption of ovarian cyclicity and the pubertal initiation of ovulation bears some similarities. Prepubertal heifers have repeated anovulatory follicular waves and as they approach puberty the interwave interval and dominant follicle diameter gradually increases (Evans *et al.*, 1994). At puberty, the first IOI is short (8 days) and is characterized by a small, short-lived CL that produces low progesterone concentrations and one wave of follicle development. The following IOI is of the same duration as that reported in adult cattle. In post parturient cattle there is a short period of repeated anovulatory follicular waves that concludes in the first post-partum ovulation. In most cattle this ovulation is followed by a short IOI (8 to 12 days) that is characterized by a small, short-lived CL that produces low progesterone concentrations and one follicular wave (reviewed by Yavas & Walton, 2000).

Future areas of study in ovarian follicle dynamics of wapiti should include the time of pregnancy and the interval preceding and following the summer solstice. In cattle, follicular waves persist during pregnancy until three weeks before parturition when follicle growth is greatly reduced (Ginther *et al.*, 1996). If follicular waves are present during pregnancy in wapiti, this may provide an opportunity for harvesting oocytes for in-vitro embryo procedures at a time that

is now unavailable for most assisted reproductive techniques. This would also have important implications on wild species. Many capture programs that are designed to tag or collect other data are conducted during the winter months when capture is facilitated by deep snow and females are usually pregnant. Techniques for the collection of oocytes could be incorporated into these programs with the objective of producing embryos, which would be stored to insure the future of the species or be used to improve the genetic diversity of captive populations.

The intervals preceding and following the summer solstice have not been studied. The present studies found differences in the maximum diameter of the dominant follicle and the length of the interwave interval at the end of the breeding season when compared to the middle of the anestrous season and the beginning of the breeding season. Further study in these areas might help characterize the rate at which these changes take place and determine whether the change in follicular diameter is a gradual process that reaches a nadir at the summer solstice or whether it is more abrupt and associated with the beginning and end of the anestrous season.

Another area of future study should involve the advances of ultrasound technology. Newer, more sophisticated equipment will have advanced functions and much higher resolution and this will continue to improve. Wapiti have proven to be excellent animals to study, in that they have tolerated the procedure very well and are generally easy to work with. They are good research animals that may help to uncover more details of ovarian function.

Recently, a study in cattle that followed the developmental pattern of small (1 mm) antral follicles found that the follicular wave began with the initial rise in FSH associated with the FSH surge (Jaiswal *et al.*, 2004). The study also suggested that selection of the dominant follicle took place much earlier than is now thought. Studying another species like wapiti will help to confirm these patterns as fundamental to ovarian function or unique to one species. Ovarian follicle dynamics have been characterized as wave-like in many species that represent several families (Bovidae, Cervidae, Camelidae, Equidae, and Hominidae). As more animals are studied, it may be established that the wave pattern of follicle development is something that is common to all placental mammals and as such, a very important and fundamental process that requires our understanding.

In conclusion, the principal hypothesis that an improved understanding of the endogenous control of ovarian function in wapiti by the characterization of follicle and luteal dynamics during the seasons of the annual cycle would lead to the development of novel methods of exogenous control that are effective and efficient has been supported by the studies reported. The knowledge of the physiology of reproduction in wapiti has been advanced providing basic information on the physiology of reproduction for all cervidae. The knowledge generated by these studies will open new paths of study and inspire new methods of exogenous control of reproductive function in cervids.

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